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Cytotoxic, apoptic and necrotics effects of *Agrocybe cylindracea* (DC.) Maire on breast cancer cells MCF-7 and L929

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Abstract
The cytotoxicity, apoptotic and necrotic effects of *Agrocybe cylindracea* were investigated.The tissue fragments from taken dry basidiocarps were developed by tissue culture method in malt extract agar.Three pellets were taken from the secondary mycelium and its development completed in 7 days at 140 rpm at 27 °C in nutrient broth.In cytotoxicity studies, sample extract concentrations were applied to MCF-7 and L929 fibroblast cells as 5mg/ml, 2.5 mg/ml, 1.25 mg/ml,0.625 mg/ml and 0.3125 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.

Key words: *Agrocybe cylindracea*, Apoptotic effect, Cancer, Cytotoxicity, Necrotic effect.

1.Introduction

Highly structured fungi (Basidiomycetes) are widely used in cancer research in the Far East, especially in traditional Chinese medicine. Mushrooms have been used as food and food-flavoring materials in soups and sauces for centuries due to their unique and subtle flavor and mushrooms have recently become attractive as functional food. In the works done; anti-tumor, anti-fungal, high cholesterol, and has functional food value for anti-depressants (Tsai et al., 2007). Using anticancer agents to progress chemotherapy to inhibit the proliferation of cancer cells is an effective means (Chien et al., 2015). In the last three decades, numerous polysaccharides and polysaccharide-protein complexes have been isolated from mushrooms and used as a source of therapeutic agents (Ooi and Liu., 2000). *Agrocybe cylindracea* (DC.) Maire is in the family of Strophariaceae (Anonymous, 2017) and it is grown in Taiwan as a black poplar mushroom (Tsai et al., 2007). It has become even more popular in recent years due to its flavor and taste (Tsai et al., 2005). In vitro cancer chemopreventive activities of polysaccharides from soybeans fermented with *Phellinus igniarius* or *Agrocybe cylindracea* (Shon and Nam 2001a). Extracts from *Agaricus blazei, Agrocybe cylindracea,* and *Boletus edulis* may have antimutagenic effects and may be effective in preventing cancer (Shon and Nam 2001b). It is thought that the methanolic extracts of *Agrocybe cylindracea* may have antioxidant properties if they protect the human body (Huang et al., 2005).

In this study, cytotoxicity, apoptotic and necrotic activity of *Agrocybe cylindracea* on breast cancer MCF-7 and L929 cancer cells were investigated.

2.Material and Method

2.1. Organism

In this study, *Agrocybe cylindracea* was used. Samples were collected from the village of Faraşlı in Sulakyurt district of Kırıkkale in 2013. The samples are stored in the Protection Biology, Mycology, Molecular Genetics Laboratory of Kırıkkale University Science and Literature Faculty with number PG590.

2.2 Cultural studies

2.2.1. Studies in solid medium

Tissue fragments obtained from the *Agrocybe cylindracea* 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 L-glutamine, 10% FCS, and 1% antibiotic and were kept in a CO_2 incubator conditioned with 5% CO_2 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 (5×10^3 cells per well) and also MCF-7 cells (5×10^3 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 CO_2 incubator (37°C in 5% CO_2) 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 *Agrocybe cylindracea* extract (5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml, 0.315mg/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 *Agrocybe cylindracea* extracts, on fibroblast cells and MCF-7 cells, were examined. Fibroblast cells (5×10^3 cells/ml) and MCF-7 cells (5×10^3 cells/ml) were cultured in separate 96-well plates in DMEM and RPMI 1640 respectively, supplemented with 10 % FCS and 1% antibiotic at 37°C and 5 % CO_2 humidified atmosphere conditions overnight. Then cells were exposed to samples of different concentrations of *Agrocybe cylindracea* (5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml, 0.315mg/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

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

Tissue fragments from the fructifications of dry *Agrocybe cylindracea* were inoculated into malt extract agar (MEA) medium and incubated in the dark for 10 d at 27°C. Mycelium began to develop after 48 h. During the development, the air hyphae observed and no pigmentation. After 10 d, colonization was completed (Figure 1).

Figure 1. *Agrocybe cylindracea* 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 (Figure 2).

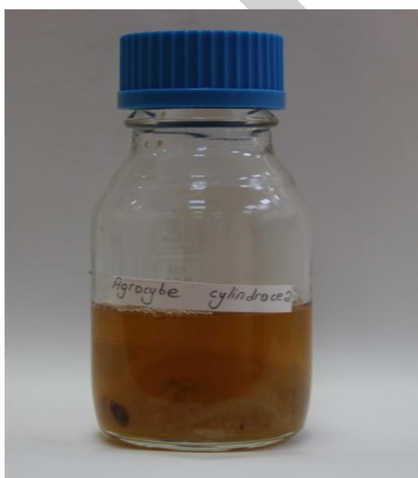

Figure 2. *Agrocybe cylindracea* on broth media.

3.2.WST assay for cytotoxicity

In this study, the cytotoxicity of the extract was tested against normal L929 fibroblast cells and MCF-7 cancer cells. The different sample concentrations were incubated with different cell lines for 24 h in cell culture media as mentioned above. In addition, the wells contain only cells specified as control were also tested in cell culture media. After that, the wells were read at 440 nm using an

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ELISA plate reader. The percentage of viable cells was calculated at different concentrations (5mg/ml, 2.5mg/ml, 1.25mg/ml, 0.625mg/ml, 0.315mg/ml) against %, cell viability (shown in Table 1). In terms of WST results, the concentration of samples didn't have a significant impact on cell mortality except 1.25-0.315 mg/ml dose of samples in both cell lines. An increase of samples concentration caused a slightly degree of cytotoxic cells in comparison to control cells. The lowest cell viability at highest concentration (5mg/ml), was seen (66.44% cell viability) on cancer cells and the lowest cell viability at highest concentration was seen (68.88% cell viability) on L929 fibroblast cells.

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

MCF-7	*Agrocybe cylindracea*	
Concentration (mg/ml)	**Absorbance (440nm)**	**Viability%**
5	1.262	66.44 ± 0.09
2.5	1.427	75.13 ± 0.02
1.25	1.883	99.17 ± 0.03
0.625	2.233	117.61 ± 0.08
0.315	2.238	117.84 ± 0.11
Control	1.899	100
L929 FIBROBLAST	*Agrocybe cylindracea*	
Concentration (mg/ml)	**Absorbance (440nm)**	**Viability %**
5	1.932	68.88 ± 0.03
2.5	2.337	83.34 ± 0.14
1.25	2.640	94.15 ± 0.02
0.625	3.124	111.41 ± 0.27
0.315	3.189	113.73 ± 0.04
Control	2.804	100

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

In this study, the apoptotic and necrotic effects associated with the morphological changes of the extracts on L929 mice fibroblast and MCF-7 cancer cells were investigated. Hoechst 33342 fluorescent stain was used in this method. For this, stock solutions of all samples were prepared and diluted for 2-16 times with fresh medium and applied into the cultured normal and cancer cell cultures. The interaction was observed for each sample with cells within 24 h period of time. The apoptotic and necrotic cells photographs based on the double-staining method are given in Figures 3. The Hoechst 33342 fluorescent dye attaches to DNA and results a dark blue color cell nuclei when observed by the DAPI filter under a fluorescent microscope. Control group contains only medium. There was no apoptosis accordingly no morphological difference in the cell nuclei of the control group. An apoptotic effect was lowest observed for the *Agrocybe cylindracea* which were different concentration compared to cells (L929 and MCF-7) treated with *Agrocybe cylindracea*, given as % apoptotic indexes in Table 1. Apoptotic cell nuclei have decomposed and have shapeless borders and a brighter appearance than the non-apoptotic cells (apoptotic cells are demonstrated with arrow in Figure 3A and C). The findings showed that lower concentrations of samples caused lower effect in apoptosis. As the concentration of samples were increased, the apoptosis rate was not also increased. PI, a fluorescent molecule was used in the double-staining method to determine necrosis in cancer cells. This dye crosses the dead cell membranes led to the cell nuclei staining red color but it does not cross the living cells under fluorescent light (by a FITC filter). The L929 fibroblast and MCF-7 cancer cells were stained with PI fluorescent dye in double-staining solution. The healthy cell nuclei appeared green when it was scanned by an FITC fluorescent filter. The control group showed no morphological difference in cell nuclei . The nuclei of necrosed cells was observed in red color in wells treated with all samples especially at high concentrations by use of a fluorescent microscope (Figure 3B and D). The lowest necrotic effect was observed at low concentration *Agrocybe cylindracea* extract and high necrotic effects were observed especially 5mg/ml concentration of samples. The necrotic effect was increased

due to higher concentrations in each type of extracts for both cell types (L929 and MCF-7). Figure 4 and Figure 5 summarize the apoptotic and necrotic cell indexes obtained from the double staining of Hoechst and PI, respectively.

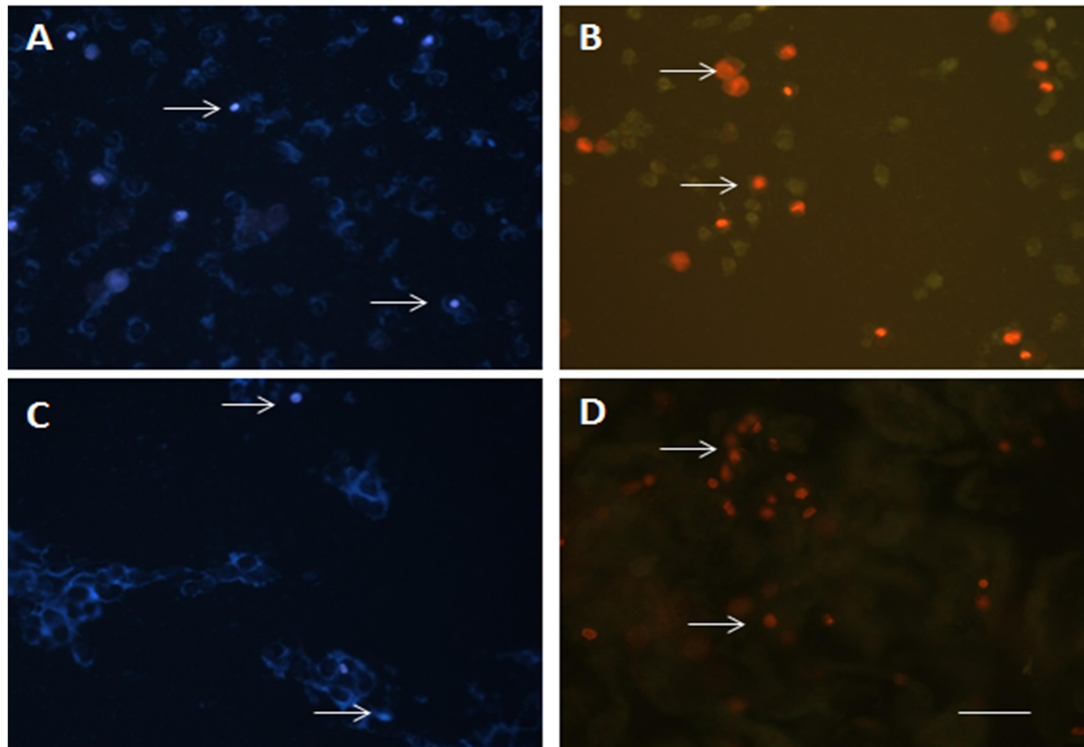

Figure 3. The apoptotic, and necrotic MCF-7 and L929 fibroblast cells under a fluorescent inverted microscope. A) Apoptotic MCF cells (white arrows) were treated with 2.5 mg/ml extract. B) Necrotic MCF cells (white arrows) were treated with 2.5 mg/ml extract, C) Apoptotic Fibroblast cells (white arrows) were treated with 2.5 mg/ml extract, D) Necrotic Fibroblast cells (white arrows) were treated with 2.5 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 (Figure 4).

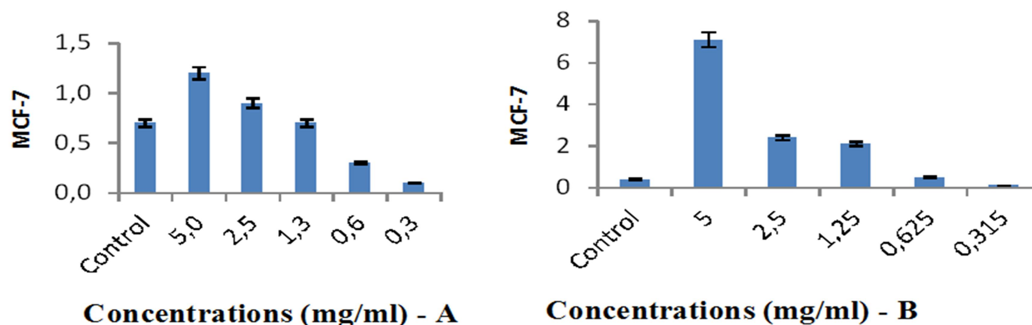

Figure 4. The apoptosis and necrosis states of the MCF-7 cells at different concentrations.
A-Apoptosis; B- Necrosis.

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

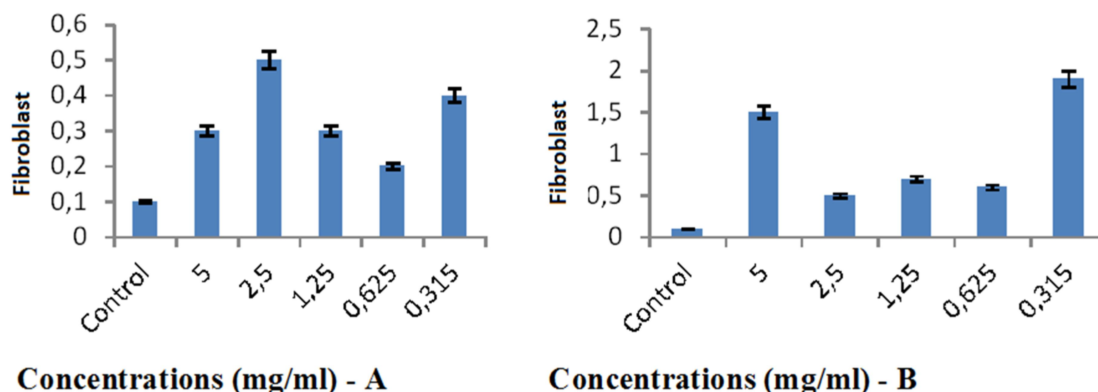

Figure 5. The apoptosis and necrosis states of the L929 Fibroblast cells at different concentrations. A-Apoptosis; B- Necrosis.

Agrocybe species are used in the medical field such as other mushrooms and especially in the treatment of cancer.

Liang et al. (2014), were investigate the clinicopathological and prognostic value of AAL in identifying aberrant glycosylation in colorectal cancer (CRC) and were determined, this is the first report about the employment of *Agrocybe aegerita* Lectin (AAL) for histochemical analysis of cancer tissues.

Two medicinal mushrooms, *Ganoderma tsugae* and *Agrocybe cylindracea,* exhibited various physiological effects, and the antiproliferation effect on HL-60, Hep 3B, and C6 cells was studied. (Chien et al., 2015). *A. cylindracea* were less effective in inhibiting the antiproliferation of C6, Hep 3B, and HL-60 cells than were those from *G. tsugae.*

Shon and Nam (2001), suggest that polysaccharides from soybeans fermented with *P igniarius* or *A. cylindracea* have cancer chemopreventive activities in in vitro models and, therefore, could be considered as potential agents for cancer chemoprevention.

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