In vitro anticancer and apoptotic activity of edible mushroom Lepista nuda (Bull.) Cooke on leukemia and breast cancer compared with protocatechuic acid, paclitaxel and doxorubicin

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Macro fungi are used as food and for therapy for centuries. One such fungus Lepista nuda (Bull.) Cooke is reported to exhibit many biological activities while other species viz. Lepista inversa and L. sordida possess active substances. In this study, we prepared extractions depended on polarity increase from the edible mushroom Lepista nuda and studied its action on HL60 (leukemia) and MCF7 (breast cancer) cancer cell lines. We used PCA (protocatechuic acid), paclitaxel and doxorubicin as positive controls. Methanol extract was found effective on both cell lines. The extract showed fairly significant (IC50 ~15 mg/mL) of biologic activity compared with drugs protocatechuic acid, paclitaxel and doxorubicin against HL-60 cell line with regard to both proliferation and apoptotic effects (>75%).

Keywords: Cancer, Cell proliferation, Macro fungi, Tumor

Cancer, as the leading cause of premature death to the tune of 10 million annually, affects the life expectancy of humans in every country. According to the Global Cancer Observatory (GLOBOCAN) 2020 report of the International Agency for Research on Cancer, World Health Organization (WHO), 19.3 million people are currently suffering from 36 types of cancers which is expected to rise to 28.4 million by 20401. In Turkey, the number of new reported cases is 0.23 million2. Globally, leukemia constitutes 2.6% of the total new cases excluding nonmelanoma skin cancer, and breast cancer accounts for 12.5%. Worldwide, 0.68 million people have died due to breast cancer and 0.31 million by leukemia1. Such serious threat to human life by cancer makes accessibility of affordable treatment for population, a basic necessity. In this context, search for new anticancer drugs which are safe and economical has geared up.

The identification of naturally occurring compounds in various living groups allows the discovery of new drugs or active substances which target cancer causing abnormal molecular and biochemical signals3,4,8. Apart from plants, mushrooms also serve as food supplements with their nutritional value and medicinal properties supporting human health.11 These fungi show antioxidant, antimicrobial and antiviral properties.12-17 Many fungal species have been studied by the researchers in terms of antioxidant content and antioxidant compounds and also have been identified in this context12,18,19. These are phenolic compounds, flavonoids, tocopherols, ascorbic acid and carotenoids20-22. Mushrooms have antioxidant properties and different flavours and attract both researchers and consumers in terms of these characteristics23,24. Mushrooms are also rich sources of anticancer compounds12,13,17,22,25-27.

Lepista nuda

Earlier researchers have reported useful bioactive constituents viz. phenolics (especially protocatechuic acid), tocopherols, ascorbic acid and carotenoids28-34 and also alcohols, aldehydes, ketones, sesquiterpene-like compounds and terpenes35 from mushrooms including Lepista nuda and also account for their antioxidant and antimicrobial effects.28,34,35 The antioxidant capacity of methanol extract from L. nuda at 100 mg/mL concentration was found to be higher than the standard compounds at 400 mg/mL.31 The linoleic acid inhibition value of L. nuda ethanol extract was found to be 84.3% (BHA: 98.9%, tocopherol: 99.2%) at a concentration of 160 μg/mL.32 Methanol extract of Lepista inversa was found to be

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effective in 4 cell lines (K562, U251, DU145, MCF7) compared with Taxol\textsuperscript{36}. \textit{Lepista nuda} mushroom extract tested on human cancer cells (HepG2, KATO III and AGS) inhibited proliferation of all three lines of cancer cells. The effect was strong between 0.5 and 1.0 mg/mL in the range of 71.4 and 91.8\%\textsuperscript{37}. In Australia, ethanol, cold and hot water extracts obtained from 15 mushrooms including \textit{Lepista nuda}, tested against normal mouse fibroblast cells (NIH/3T3), healthy human renal epithelial cells (HEK293) and 4 cancer cell lines (AGS, MDA-MB-231, MCF7, HT29) using MTT method. Extract of \textit{Lepista nuda} were found to be effective against 1 or 2 cancer cell lines\textsuperscript{38}. A new Metalloprotease isolated from \textit{Lepista nuda} has been tested in hepatoma (HEPG2) and leukemia (L1210) cancer cell lines. IC\textsubscript{50} values were found to be 4.99 µM and 3.67 µM, respectively\textsuperscript{39}.

\textit{Lepista nuda} is reported to contain active ingredients such as PCA (Protocatechuic acid), other phenolics, carotenoids and terpenes and will show biological activity against cancer cells. Here, we tested extracts from \textit{Lepista nuda} mushroom on two cancer cell lines (HL60, MCF7) using MTT method to study the effects of antiproliferative activity and apoptosis. The results were compared with the known drugs paclitaxel and doxorubicin and with PCA (Protocatechuic acid) as active ingredient.

**Materials and Methods**

**Mushroom material**

The mushroom \textit{Lepista nuda} (Bull.) Cooke (Fam.: \textit{Tricholomataceae}) was collected from the pine forest and open fields of Yerlisu köyü and Karatepe mevki, Keşan, EDİRNE, northwest of Turkey on 2\textsuperscript{nd} and 3\textsuperscript{rd} of November 2016. This mushroom was identified in the field survey on comparison with previously prepared herbarium samples. Taxonomic determination was made by Dr. Hakan ALLI from Muğla Sıtkı Koçman University. Voucher specimens, in duplicates were deposited in the herbarium of the Department of Biology, Aydın Adnan Menderes University. The others were stored at −80\(^\circ\)C.

**Drying and extraction**

Mushrooms were freeze dried by lyophilisation, and then the material was milled; the obtained material was weighed and extracted in a solvent series of increasing polarity (petroleum ether, dichloromethane, ethyl acetate and methanol)\textsuperscript{40}. Solvent was added 1:10, (e.g. 10 g material to 100 mL petroleum ether). After finishing the first extraction with petroleum ether, the obtained residues were filtered and the dried material was subjected sequentially to second extraction with dichloromethane, the third extraction with ethyl acetate, and fourth extraction with methanol. Extracts were prepared using soxhlet and rotary evaporator apparatus, which yielded dried extracts.

**Dissolving of extracts**

After evaporation of the solvents, the dried extracts were taken from the rotary evaporator and weighed. The amounts from the extracts, corresponding to 10 g dry weight of the mushroom (before extraction) were dissolved in 1.0 mL ethanol\textsuperscript{40}. For proliferation assay, concentrations of 10, 20 and 40 mg/mL were used.

**Cell culture**

HL60 leukaemia cells and MCF7 breast cancer cells were purchased from ATCC. Cells were grown in RPMI 1640 and DMEM mediums supplemented with 10\% heat inactivated fetal calf serum, 1\% L-glutamine and 1\% penicillin/streptomycin at 37\(^\circ\)C in a humidified atmosphere containing 5\% CO\textsubscript{2}. All media and supplements were obtained from Life Technologies. Hoechst 33258 and propidium iodide were purchased from Sigma.

**Proliferation inhibition assay**

HL-60 and MCF-7 cells were seeded in 24-well plate at a concentration of 1×10\(^6\) per mL and incubated with increasing concentrations of extracts (10, 20 and 40 mg/mL), drugs (doxorubicin 0.5 µM, paclitaxel 0.5 µM) and PCA (5 µM). Cell counts and IC\textsubscript{50} values were determined for 48 h using MTT [3 (4,5-dimethyl thiazol 2 yl) 2,5-diphenyl tetrazolium bromide] staining assay. After 4 h incubating period with 5 mg/mL MTT-PBS staining solution, 150 µL of MTT solving solution were added per well. The OD values were measured using plate reader at 590 nm wavelength. Experiments were done in triplicate and the percentages of living cells were calculated as follows\textsuperscript{41-43}. For control: mean OD control/mean OD control *100 =100%. For treatment: mean OD treatment/mean OD control *100= Living cells%

**Hoechst 33258 and propidium iodide double staining**

The Hoechst staining was performed according to the method described by Grusch \textit{et al.}\textsuperscript{44}. HL-60 and MCF-7 cells (1×10\(^6\) per mL) were seeded in 24-well plate and incubated with 40 mg/mL concentration of extract, drugs (doxorubicin 0.5 µM, paclitaxel
0.5 µM) and PCA (5 µM) for 48 h. Hoechst 33258 and propidium iodide were added directly to the cells at final concentrations of 5 and 2 µg/mL, respectively. After 60 min of incubation at 37°C, cells were examined on Olympus BX51 fluorescence microscope equipped with a DAPI filter. Cells were photographed and analysed by visual examination (not by FACS). This method allows to distinguish between apoptosis and necrosis\textsuperscript{44,45}. Cells were judged according to their morphology and the integrity of their cell membranes, which can easily be seen upon propidium iodide staining.

Results and Discussion

The study was carried out in two basic stages which determine the activity of inhibiting the proliferation and determining the apoptotic effect. In this context, extracts were obtained from collected fungi. Subsequent antiproliferative activities were determined using the HL60 human leukaemia cell line and MCF7 breast cancer cell line and concentration of the most effective extract type inhibiting 50% of the cells was selected. Apoptotic effects were determined with selected extract type. The findings of the study are presented below in the order listed above.

Antiproliferative activity

The proliferation data obtained as the result of experiments as detailed under methods are presented graphically in the Figs 1 and 2). Fig. 1, shows the effect of all the tested drugs and extracts in different concentrations against HL60 cell line. Doxorubicin and paclitaxel are well known cancer drugs and at 0.5 µM these drugs have killed more than 50% of the cells. Also, PCA at 5 µM have destroyed more than 50% of the cells. The best results with the mushroom extracts were obtained from ethyl acetate, dichloromethane and methanol solvents and these effects were found also statistically important. But they were effective at high concentrations. Only methanol extract wiped out more than 50% of cells between the 10 and 20 mg/mL concentration range. If compared with the known drugs and PCA the methanol extracts was found as the nearest active extract to the drugs and PCA. Therefore, it can be suggested that the active compounds of the mushroom can be dissolved in methanol better than the other solvents.

Figure 2 shows the effects of all the tested drugs and extracts in different concentrations but differently against MCF7 cell line. Similarly, doxorubicin, paclitaxel and PCA destroyed more than 50% of the cells at applied concentrations. In MCF7 cell line results, the most effective extract type was found as methanol at the high concentration (40 mg/mL) and the effect was weaker than the effect on HL60 cell line.

The next question was about how the cells died after application of drugs and extract types. The methanol extract proved to be the most effective extract type for comparing the cell death type between two cell lines HL60 and MCF7 the same concentration (40 mg/mL). In Fig. 3, we could

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**Fig. 1** — *Lepista nuda* antiproliferative activity on HL-60 cell line. [Extracts of *Lepista nuda* mushrooms were applied in increasing concentrations to HL-60 cells. Among the extracts tested in the range of 10-40 mg/mL, the most effective was found to be methanol extract. The IC\textsubscript{50} value of the methanol extract was calculated to be approximately 15 mg/mL.]

**Fig. 2** — *Lepista nuda* antiproliferative activity on MCF-7 cell line. [Extracts of *Lepista nuda* mushrooms were applied in increasing concentrations to MCF-7 cells. Among the extracts tested in the range of 10-40 mg/mL, the most effective was found to be methanol extract. The IC\textsubscript{50} value of the methanol extract was calculated to be approximately 40 mg/mL.]
observe apoptosis and necrosis ratios after 48 h application of the known drugs, PCA and 40 mg/mL methanol extracts in both cell lines. By comparison with the known drugs, the observed apoptosis ratios after extract application were found statistically important and especially the results of HL60 cell line showing significantly strong apoptotic effect of methanol extract.

The cell lines studied by Bézivin et al.36 and our study here are the same. The MCF7 is used as a joint, while K562 is another leukemia cell line such as the HL60. In our study, the concentration that inhibited about 50% of the cells was 15 mg/mL for HL60 and 40 mg/mL for MCF7 (Figs. 1 and 2). On the other hand, taxol was preferred as positive control by Bezivin et al.36 and it was found that Lepista inversa methanol extract was more effective in the treatment of cancer. Similarly, in our study, methanol extract was found to be more effective than doxorubicin and paclitaxel. Elmastaş et al.31, Bezivin et al.36 and Sanchez46 have reported that most of the compounds are obtained from the methanol extract of Lepista nuda. Furhter, Sanchez46 also reported the presence of β-carotene and α-tocopherol in the L. nuda methanol extract. Similarly, Elmastaş et al.31 have also obtained the antioxidant compounds from methanol extract of L. nuda.

In this study, we used protocatechuic acid (PCA) as active ingredient. Barros et al.33 which analysed the flavonoids and phenolics contents of 16 different fungus species reported that Lepista nuda contains 6.31 mg/g total phenolics and 33.47 mg of protocatechuic acid (PCA) in 1.0 kg dry material. PCA is reported to inhibit proliferation and have apoptotic effects on cancer cells. These effects of PCA have also been studied in human leukemia and human breast cancer cells. Tseng et al.47 studied the effects of PCA in human leukemia cells (HL60). Approximately, 50% of the cells died in 48 h and apoptotic effects were observed in 46.7% of the cells. In another study48, the apoptotic effect of PCA was determined in human breast cancer cells MCF7. Xie et al.49 revealed that PCA could modulate apoptosis and autophagy suggesting the potential of PCA for chemoprevention and chemotherapy of ovarian cancer.

Apoptosis removes malignant or cancer cells from normal cells or tissue surrounding them without damage. Disorders in the function of apoptosis are common in many types of cancer and are resistant to treatment. Therefore, apoptosis pathways are the main targets for cancer treatment50. Fortin et al.51, isolated clitocin from Lepista inversa and tested in human cancer cell lines (DU145, K562, MCF7 and U251). They found clitocin to be effective in the concentration range of 185-578 nM. Further, the flow cytometric analysis revealed that clitocin antitumor activity was associated with apoptosis stimulation51. Although there are no studies on the apoptotic activity of Lepista nuda, the efficacy of the Lepista inversa active agent clitocin is a reference to our study. From Lepista sordida species a polysaccharide having a potent antitumor effect (LSPc1) was obtained. The anticancer activity and the mechanism of action of this substance were studied on laryngeal cancer (Hep2) cell line. The agent LSPc1 inhibited cell cycle by accumulating cells in G2/M phase within 48 hours. Additionally, apoptotic effects were observed morphologically and biochemically52. In this study, more than 75% apoptotic cells were observed, especially in the HL60 cell line for 48 h (Fig. 3). While the doxorubicin from the used positive controls, leads to cell death by necrosis, paclitaxel causes apoptosis. If compared as extract and pure drug the methanol extract was found to be more effective than paclitaxel on HL60 cell line and the act of mechanisms seems to be the same with PCA.
Conclusion
When the results obtained from our study are evaluated together with literature data, it is obvious that there are compounds showing anticancer properties in Lepista fungi. Additionally, their activities on the cell lines are similar. The relevant active ingredients primarily stop the cell cycle; subsequently they induce stimulation of the apoptotic pathway. Morphological and biochemical results support this. Although there is not much work on this subject, in light of the data obtained from our study, it can be suggested that the compound or compounds found in Lepista nuda mushroom that are responsible for biological activity have the effect of stimulating apoptotic cell death together on HL60 and MCF7 cell lines. This activity is important in terms of molecular mechanisms targeted in cancer treatment approaches.

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Conflict of interest
Authors declare no conflict of interests.

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