Cytotoxic and Apoptotic Activities of *Rhizopogon roseolus* (Corda) Th.Fr. Extracts

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**Abstract:** Many species of mushrooms have been used since ancient times, especially in Asian countries, as a food supplement and in the medical field due to their different biological activities. Nowadays, especially in Japan, Korea and China, various mushroom extracts have been used as potential additives in chemotherapy and radiation treatments. In this study, anticancer activity and apoptotic effect of *Rhizopogon roseolus* were investigated. The methanol and water extracts of mushroom were tested against HL-60 human cancer cell line. Antiproliferative effects of the extracts were evaluated by using MTT method and apoptosis and necrosis ratios of the cells treated with extracts were determined by using Hoechst/Propidium iodide (HO/PI) staining method. According to obtained data, antiproliferative effect of the methanol extract was higher than water extract and this effect was a concentration depending manner. Both of the extracts were shown higher apoptotic effect than necrotic effect on the HL-60 cell line.

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1. INTRODUCTION

Edible mushrooms are widely consumed in many countries because of their low calorie nutrients and specific aromas [1,2]. According to data from the United Nations Organization for Food and Agriculture (FAO), worldwide production of mushroom has been increased by about 73% from 5.91 million tons in 2007 to 10.24 million tons in 2017 [3].

Edible mushrooms are rich in high minerals (potassium, phosphorus, iron), essential amino acids, vitamins (B12 and D) and source of some fiber [1,4,5]. Mushrooms are very attractive in food and pharmaceutical researches due to their bioactive components, such as phenolic compounds, terpenes, steroids and polysaccharides, that have a variety of biological activities. [6,7,8]. The mushroom’s compounds possess antifungal activity [9], antigenotoxic [10], antioxidant [5], antiproliferative [11], anticancer [12], anti-hyperlipidemic [13], anti-hypertensive, anti-nociceptive and immunostimulating [14], hypocholesterolemic, anti-atherogenic [15], and stress reducing properties [16]. In addition, edible mushroom are good sources of prebiotic substances, especially those containing short chain sugars such as glucose,

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galactose, fructose and N-acetylglucosamine. These include highly bioactive polysaccharides and digestible carbohydrates that stimulate the growth of beneficial microorganisms [17]. These microorganisms serve as probiotics because of their potential to inhibit pathogenic microorganisms in the gastrointestinal tract. In addition, prebiotic compounds have gastrointestinal tolerant in the presence of amylase, gastric juice or bile extract in saliva. Therefore, they afford the ability to activate germs useful for host health. Also, they can be health supportive [18]. In recent years, antidiabetic effects of Agaricus blazei, Coprinus comatus, Cordyceps militaris, Inonotus obliquus, Morchella conica macrofungi have been demonstrated [19].

Strong anti-cancer activities of extracts and bioactive components isolated from different mushrooms have become increasingly understandable [20]. The study received in 1970 was related to pharmacological activities reported that Lentinus edodes and Agaricus bisporus were effective against cancer cells and this work has been an inspiration source for the further discovery of effective molecules [21,22].

The most important ectomycorizal fungi are sulloid fungi. The truffle-like Suillus and Rhizopogon genera are the largest ectomycorizal groups [23]. R. roseolus is a fungi species that establishes an ectomycorizal relationship with the Pinaceae family [24]. The species was first described in Europe in the 19th century (25). This mushroom has been involved in numerous research programs targeting afforestation. It is also an exemplary species for morphology, physiology and ecology in ectomycorrhizal relationships [26]. A literature review showed that several biological activity of R. roseolus were identified. Akata et al., investigated antioxidant properties of R. roseolus. The high antioxidant activity at low concentration of extract was shown [16]. Yamaç et al., studied antimicrobial activities of ethanol and dichloromethane R. roseolus extracts. It was found, the ethanol and dichloromethane extracts have a low antimicrobial effect against Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Staphylococcus aureus (ATCC 25923); chloroform extract against Pseudomonas aeruginosa (ATCC 27853) and Staphylococcus aureus (ATCC 25923) [27].

Cancer was one of the leading cause of global deaths in 2018, responsible for 18.1 million new cases and 9.6 million cancer deaths worldwide [28]. The incidence and mortality of cancer have been increasing rapidly for decades due to environmental violations and socioeconomic developments deteriorating [29]. The survival rate of cancer patients has been extended due to recent advances in the diagnosis and treatment of diseases [30].

The definition of traditional medicine according to World Health Organization; plant, animal and mineral based health practices are described as studies aimed at treating or maintaining health [31]. Metabolic syndromes affect people of all age groups. Natural compounds are noteworthy because chemical compounds are perceived to be incompatible with the human body. Depending on the stage of cancer progression, the treatments include surgical operation, radiotherapy, chemotherapy, and biological therapy. Existing anti-cancer chemotherapeutic agents are formulated with toxic solvents and this situation results in various side effects and complications in the clinical management of various forms of cancer. When administered, these drugs cause significant damage to non-cancerous tissues. This usually leads to serious and unwanted side effects, such as kidney toxicity, bone marrow suppression, hair loss (alopecia) and grease of intestinal epithelial cells. Therefore, the search for new and natural anticancer bioactive compounds has become of great interest [32]. In this study, the antiproliferative and apoptotic effects of methanolic and water extracts of mushrooms R. roseolus on the HL-60 cancer cell line were investigated firstly.
2. MATERIAL and METHODS

2.1. Mushroom Material

*R. roseolus* was collected from Bozkır district of Konya in 2012 and was identified by Prof. Dr. Hasan Hüseyin Doğan from Selçuk University.

2.2. Preparation of the Extract

*R. roseolus* powder was extracted by ten fold methanol and water for one week at room temperature and dark environment. After filtration through a Whatman filter paper, the solvent was removed with a rotary evaporator at 70 mbar at 45°C to give a solid extract. Dissolved extracts were centrifuged at +4°C in a refrigerated centrifuge for 5 minutes at 12,000 rpm. The resulting supernatants were transferred to another tube for cytotoxic evaluation and the remaining pellets were stored at -80°C.

2.3. Cell Culture

The HL-60 cell line was obtained from American Type Culture Collection (ATCC), and it was been grown in Dulbecco’s Modified Eagle’s Medium (DMEM) (invitrogen). The culture medium additions [fetal calf serum, L-glutamine, streptomycin-penicillin, non-essential amino acids (GIBCO), Hoechst 33258, and propidium iodide (Sigma-Aldrich Co.)] were used to determine apoptotic effect.

2.3.1. Antiproliferative Activity of the Extract

Cells were grown in culture flask at a range of 10,000-100,000 cells per ml. Mushroom extracts were applied at increasing concentrations (1, 5, 10, 20 and 40 mg/mL) for 24, 48 and 72 hours. Viable cells in the control and application groups were determined by MTT [3-(4,5-dimethyl thiazol-2-yl) -2,5-diphenyl tetrazolium bromide] staining method [33]. The solution was measured by spectrophotometer (Thermo/LabSystems 352 Multiskan MS Microplate Reader) at 590 nm.

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[(C_{72h+extract} − C_{24h+extract}) / (C_{72h−control} − C_{24h−control})] \times 100 = \% \text{ dividing cell viability}
\]

- C_{72h+extract}: Live cell measurement 72 hours after manipulation
- C_{24h+extract}: Live cell measurement 24 hours after manipulation
- C_{72h−control}: 72 hours after live cell measurement without extract manipulation
- C_{24h−control}: 24 hours after live cell measurement without extract manipulation

All experiments were performed with three replications.

2.4. Apoptosis and Necrosis Effects of the Extracts

For determination of the apoptotic and necrotic effects the HL-60 cells were grown at low density in culture flasks (DMEM). These cells were stained with HO/PI (Hoechst 33258/Propidium Iodide) method and extracts showing apoptotic and necrotic effects were determined [34,35]. These cells were investigated and were counted under microscope (Leica). Graphics are presented in comparison with the control group. Apoptosis and necrosis rates were expressed as % increase (% control) in the application groups compared to the number in the control group.

2.5. Statistical Evaluation

Differences between control and application groups were transferred to graphics containing standard errors with GraphPad 4.0 analysis program. The living cells in the control group and the living cells in the application groups are shown in separate columns. Standard errors in the data obtained in three replicates were calculated.
3. RESULTS and DISCUSSION

3.1. Antiproliferative Activity of the Extracts

The experimental data of antiproliferative activity of *R. roseolus* extracts on HL-60 cell line by MTT method are shown in Figure 1. It was found, that the methanol extract in the range of 1-40 mg/mL have a better antiproliferative activity of HL-60 cell line than the water extract of *R. roseolus*. In addition, the lowest concentration of methanol extract 1mg/mL of methanol extract on HL-60 cell line which is the lowest concentration of caused less than 50% of the antiproliferative effect was found to be with high statistical significance. Furthermore, the value at which the methanol extract kills all the cells is 5 mg/mL, while the water extract is approximately 16 mg/mL.

![Figure 1](image1.png)

**Figure 1.** Influence of *R. roseolus* extract on the proliferation of HL-60 cell line (C: Control, M: Methanol)

3.2. Apoptotic and Necrotic Effects of the Extracts

As a result of the counts and analyzes, the findings were shown in Figure 2 and Figure 3.

![Figure 2](image2.png)

**Figure 2.** Percentages of the apoptosis and necrosis of the cells treated with the methanol extract during 24 and 48 hours (C: Control, M: Methanol).

Approximately 50% apoptotic effect was observed with 24 hours incubation of *R. roseolus* 5 mg/mL methanol extract on HL-60 cells. Moreover, this rate increased to approximately 75% with 48 hours incubation. Besides this apoptotic effect, the necrotic effect has hardly been observed. As the concentration of the extract increased, more apoptotic activity was observed.
In particular, the extract of 40 mg/mL concentration was found with 48 hours incubation 100% apoptotic activity.

Figure 3. Percentages of the apoptosis and necrosis of the cells treated with the water extract during 24 and 48 hours

The water extract of *R. roseolus* increased in parallel with the increase in apoptotic effect concentration. Treatment with water extract inhibited the growth of HL-60 cell in a time dependent manner and the concentration of between 20-40 mg/mL against HL-60 was found with 48 hours incubation the best apoptotic activity (approximately 70%), but the necrotic effect has hardly been observed.

Reishi mushroom is an important medicinal mushroom in traditional Chinese medicine more than two thousand years. The most widely used species of Reishi mushroom is *Ganoderma lucidum*, which is currently commercially grown. *G. lucidum* (F.) Karst. (Polyporaceae) is used in the prevention and treatment of various diseases such as hypertension, tumorogenic diseases and immunological disorders in China, Japan and other Eastern countries. Although the fruit body of *G. lucidum* has been used as a traditional herbal remedy since ancient times, the spores have been started to use in the late 20th century [36-40]. In the study of Hu et al., demonstrated that cytotoxic activity of *G. lucidum* alcohol extract on the MCF-7 cancer cell line. After 48 hours, at 500 μg/mL concentration, this alcohol extract caused about 70% inhibition of cell growth compared to the control [41]. In another study, Kim et al. [42] investigated the cytotoxicity of *G. lucidum* extract on HL-60 cells by conventional tetrazolium-based colorimetric cell proliferation assay. After 48 hours incubation, they reported that 210 μg/mL required to kill 50% of the cells (IC<sub>50</sub>) [42].

Some fungi belonging to the genus *Suillus* have significant medical activities [43,44]. Santos et al., investigated that antiproliferative activity of *S. luteus* methanol extract on colon cancer cell line by MTT method. The most effective amount was found to be IC<sub>50</sub> = 17.75 ± 1.6 μg/mL on HCT-15. They also investigated its apoptotic effect on lung cancer cell line p-H2A.X by TUNEL method. They found the IC<sub>50</sub> values in 24 and 48 hours as 1.2 ± 0.4 and 1.2 ± 0.06 mg/mL, respectively [45]. Vaz et al., also concluded cytotoxic activity of *S. collinitus* on ASG gastric cancer cell line. They found that the cell line had IC<sub>50</sub> value with 79.2 ± 15.5 μ/mL [14].

*Hericium erinaceus* is a fungus consumed as food. In recent years, novel compounds with multifaceted bioactivities, such as isoindolines and diterpenoids, have been discovered in this fungus [46-49]. In previous studies, Chen et al., [50] reported that cytotoxic activity of diterponoid isolated *H. erinaceus* on HL-60 cancer cell line was determined. They found the IC<sub>50</sub> on the HL-60 cell line to be 8.9μ/mL [50]. Lavi et al., [51] found that antiproliferative activity of *Pleurotus ostreatus* crude extracts on HT-29 colon cancer cells. According to these
results, the IC$_{50}$ value of ethyl acetate extract was found as 0.05 mg/mL and IC$_{50}$ value of n-hexane extract was found as 0.2 mg/mL [51]. The different fungi studied under the research have differences both at the cell line level and on excitation of apoptosis mechanisms. These differences are linked to the active ingredients contain.

4. CONCLUSION

*R. roseolus* samples have high cytotoxic and apoptotic activity at low concentration of methanol and water extracts. In conclusion, our studies proved that *R. roseolus* extracts of the HL-60 cells in a dose-dependent manner as well as apoptosis. Cytotoxic activity studies of *R. roseolus* have not been encounter in the literature. Further studies can be conducted on *R. roseolus*, especially methanol extract as a potential anticancer agent. It is predicted that these new findings added to the literature will be effective in further studies.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

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