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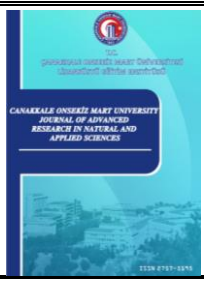

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Effects of *Pleurotus Eryngii* (DC.) Quél. Mushroom Extracts on Cell Proliferation in Breast and Colon Cancer Cell Lines

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Abstract − This study was aimed to collect *Pleurotus eryngii* (DC.) Quél. edible/medicinal mushroom from Çanakkale/Kumkale and to evaluate the antiproliferative effects of alcoholic extracts on breast and colon cancer cell lines. For this purpose, the Soxhlet method prepared methanol and ethanol:H2O (70:30) extracts by gradually increasing solvent polarities. Then, breast (MDA-MB-231, BT-549, BT-20, MCF-7) and colon (HT-29) cancer cell lines were treated with the extracts in increasing concentrations (0.05-0.5 mg/mL) for 48 h. In conclusion, methanol and aqueous ethanol extracts exhibited significant antiproliferative effects in cancer cell lines, according to the MTT assay. The cell viability in the triple-negative breast cancer (TNBC) MDA-MB-231 cell line was highly decreased by methanol extract at a very low concentration (0.1 mg/mL). Furthermore, methanol extract at 0.3 mg/mL reduced the percentage of cell viability in the HT-29 cell line. Aqueous ethanol extract showed antiproliferative activity in breast and colon cancer cell lines at 0.25 mg/mL concentration for 48 h applications. In addition, some bioactive components such as 4-hydroxy flavone, chrysin, and tannic acid of aqueous ethanol extract have been determined by High-Performance Liquid Chromatography (HPLC) analysis. As a result, this study may increase interest in the studies on the anticancer activities and the mechanisms of *P. eryngii* mushroom.

Keywords − *Pleurotus eryngii* (DC.) Quél., *mushroom, antiproliferation, natural products, cancer*

1. Introduction

There are many natural resources, such as edible/medicinal mushrooms in nature for human health. The use of fungi as medicine and food has existed since time immemorial. Since fungi have chitin in their cell walls, they are included in an independent class called Mycota [1]. More than 14,000 species of mushrooms exist in nature, and 2,200 of them are edible [2]. Approximately 650 species are known for health and medicinal usage [2]. The fungal cell wall contains chitin, glucans, and glycoproteins [3]. Moreover, fungi contain bioactive compounds such as polyphenols, terpenoids, polysaccharides, and proteins [4]. Fungal compounds fight cancers by modulating various immune systems [5]. Since cancer-related deaths are estimated to increase to 13 million by 2030, efforts to find effective and better treatments to fight the disease are increasing [6]. Nevertheless, modern anticancer therapy causes various adverse effects, including impairment of the immune system [7, 8]. Nowadays, there is a need for alternative therapies to regulate cancer cells and, at the same time,

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reinforce the immune system [5]. It was reported that mushroom extracts exhibited anticancer effects and positively affected human health [9].

Several edible/medicinal mushrooms have been reported for their anticancer effects *on breast cancer in vitro and in vivo* and evaluated for their mechanisms [9]. Furthermore, several mushrooms were studied in Phase clinical trials to treat breast, colon, and prostate cancers. It was reported that most *in vitro* studies were performed on breast, lung, and colon cancer cell lines [10]. Considering these explanations, mushrooms have been reported to be associated with the treatment of various types of cancer, including breast, colorectal, ovarian, stomach, skin, lung, cervical, liver, bladder, prostate, and leukemia [11].

Pleurotus species have been reported for their medicinal properties, such as anti-tumor, hepatoprotective, and hypolipidemic [12-14]. *P. eryngii* is a well-known edible/medicinal mushroom [12, 15-18]. *P. eryngii* grows in three villages of Türkiye (Muğla, Çanakkale, and İzmir). *P. eryngii* was called "körek mushroom" in the Egea region and "çakşır mushroom" in Anatolia of Türkiye [19]. In one of the reported studies, *P. eryngii* collected from Muğla was conducted for its fatty acid content [19]. In another study, its extracts were studied on bone metabolism [20]. Moreover, the researchers reviewed its role in biotechnological processes [21]. In the previously reported study, the *P. eryngii* mushroom was used for the mycosynthesis of silver nanoparticles in biomedical applications. Then, AgNPs were analyzed for deoxyribonucleic acid (DNA) damage, antioxidant activity, and lipid peroxidation [22].

In this study, it was the first time, *P. eryngii* mushroom was collected from Çanakkale/Kumkale of Türkiye. This study aimed to investigate *in vitro* antiproliferative effects of the alcoholic extracts of mushrooms on various cancer cell lines. Therefore, methanol and aqueous ethanol extracts of *P. eryngii* mushroom were gradually obtained by Soxhlet extraction. Then, these extracts were applied to breast and colon cancer cell lines to reduce cell proliferation. According to our findings, the proliferation of various cancer cells decreased in the increasing doses of the applications in dose- and time-dependent manner studies.

Figure 1. *P. eryngii* mushroom collected from Çanakkale/Kumkale (The photos were taken by G.D.)

2. Materials and Methods

2.1. Materials

All solvents, including *n*-hexane, ethyl acetate (EtOAc), methanol (MeOH), ethanol (EtOH), and dimethyl sulfoxide (DMSO), were obtained from Sigma and Merck with HPLC grade. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Cayman Chemical. DMEM/F12 (Dulbecco's Modified Eagle Medium) (Thermo, Gibco), Penicillin-streptomycin (PS) (Thermo, Gibco), fetal bovine serum (FBS) (Serox), and 0.25% trypsin-EDTA (Thermo, Gibco) were used. A rotary evaporator (IKA RV10) and a microplate reader (Thermo) were used in the experiments.

2.2. Collection and Identification of *P. Eryngii* Mushrooms and Preparation of its Extracts

P. eryngii mushroom was collected from Çanakkale-Kumkale. Then, the mushrooms were identified and kept at the Department of Biology Muğla Sıtkı Koçman University in Fungarium. The edible mushrooms were divided into small pieces, and water was removed at 50-60 °C. The dried samples were taken into the grinder. The extraction process was carried out by the traditional Soxhlet method. At this stage, it was carried out gradually with the help of various solvents. The extraction was carried out with *n*-hexane, EtOAc, MeOH, and EtOH: water (EtOH:H_2O) (70:30) solvents, respectively. Then, a rotary evaporator was used to remove the solvents at reduced pressure under vacuum. The obtained extracts were dried at room temperature. Then, it was weighed and stored in the refrigerator at +4 ºC. The freshly prepared intermediate solutions were prepared from stock solutions (10 mg/mL) before use in *in vitro* cell culture assay [23].

2.3. Cell Culture Conditions

The human breast (MDA-MB-231, BT-549, BT-20, MCF-7) and colon (HT-29) cancer cell lines were used in cell culture studies. The cells were incubated in DMEM/F12 at 37ºC in an incubator containing 5% CO_2. PS (1%) and FBS (10%) were used. Passaging was performed using 0.25% trypsin-EDTA at appropriate passage times [23].

2.4. Antiproliferative Activity

To determine the antiproliferative effects of the mushroom extracts on different cancer cell lines, MTT assay was used according to a reported study [23]. A representative workflow is shown in Figure 2. Thus, this assay was applied for 48 h treatments in the increasing dose-dependent manner studies. After incubation, the MTT solution (10 µL) was added and incubated for 3-4 h in an incubator at 37 ºC. The absorbances at 570 nm were measured in a 96-well plate following the addition of dimethyl sulfoxide (DMSO) (100 µL). The percentage of cell viability (%) graphs were formed against DMSO as a control. Statistically significant results were taken and indicated with an asterisk [23].

2.5. HPLC Analysis

30% of Aqueous ethanol (EtOH) extract was used in this analysis. The detailed instrument conditions and injection volume, flow rate, solvent system, wavelengths, and standards were given in a previously reported study [23].

2.6. Statistical Analysis

Statistical analysis was performed using the Welch t-test of the GraphPad Prism program. Significant results of $p < 0.05$ were evaluated.

Figure 2. A representative workflow for this study

3. Results and Discussion

3.1. *P. Eryngii* Mushroom Extracts Show Antiproliferative Effect on Human Cancer Cell Lines

This study tested the antiproliferative effects of methanol (MeOH) and aqueous EtOH extracts obtained from *P. eryngii* mushroom by MTT assay. In the increasing concentrations (0.05-0.5 mg/mL), their effects on short-term cell proliferation against MDA-MB-231, BT-549, BT-20, MCF-7, and HT-29 cell lines were evaluated. According to our findings, it has been determined that MeOH extract significantly decreased the cell viability of MDA-MB-231 cells at 0.1 mg/mL for 48 h treatments (Figure 3a). Unlike this, it was determined that MeOH extract showed an antiproliferative effect on the HT-29 cells at 0.3 mg/mL (Figure 3e). The effect of the MeOH extract on TNBC BT-549 and BT-20 cell lines exhibited similar results at 0.1 mg/mL concentration of the extract (Figure 3b,c).

Although aqueous EtOH extract displayed a significant antiproliferative effect on breast cancer cells between 0.125 mg/mL and 0.5 mg/mL concentrations. (Figure 4a-d). It was determined that the cell viability of MDA-MB-231 cells was decreased with the treatments of the aqueous EtOH extract at 0.25 mg/mL (Figure 4a). IC_{50} values were obtained at 0.375 mg/mL and 0.25 mg/mL on BT-549 and BT-20 cell lines, respectively (Figure 4b,c). The percentage of cell viability was significantly reduced on MCF-7 cells at 0.25 mg/mL, as shown in Figure 4d. Moreover, in Figure 4e, the extract was highly cytotoxic on HT-29 cells at 0.25 mg/mL concentration.

According to previously reported studies of the mushroom extracts, water extracts of the fruiting bodies of *Agaricus blazei* Murill (AbM), a species of mushroom, induce interleukin-8, TNF-alpha, and nitric oxide production, and it was also reported to suppress tumor growth *in vivo* [24]. *Ganoderma lucidum* inhibited

tumor growth in *in vitro* and *in vivo* studies [25]. In addition, *G. lucidum* and *G. tsugae* extracts have been reported to inhibit the growth of colorectal cancer cells *in vitro* [26].

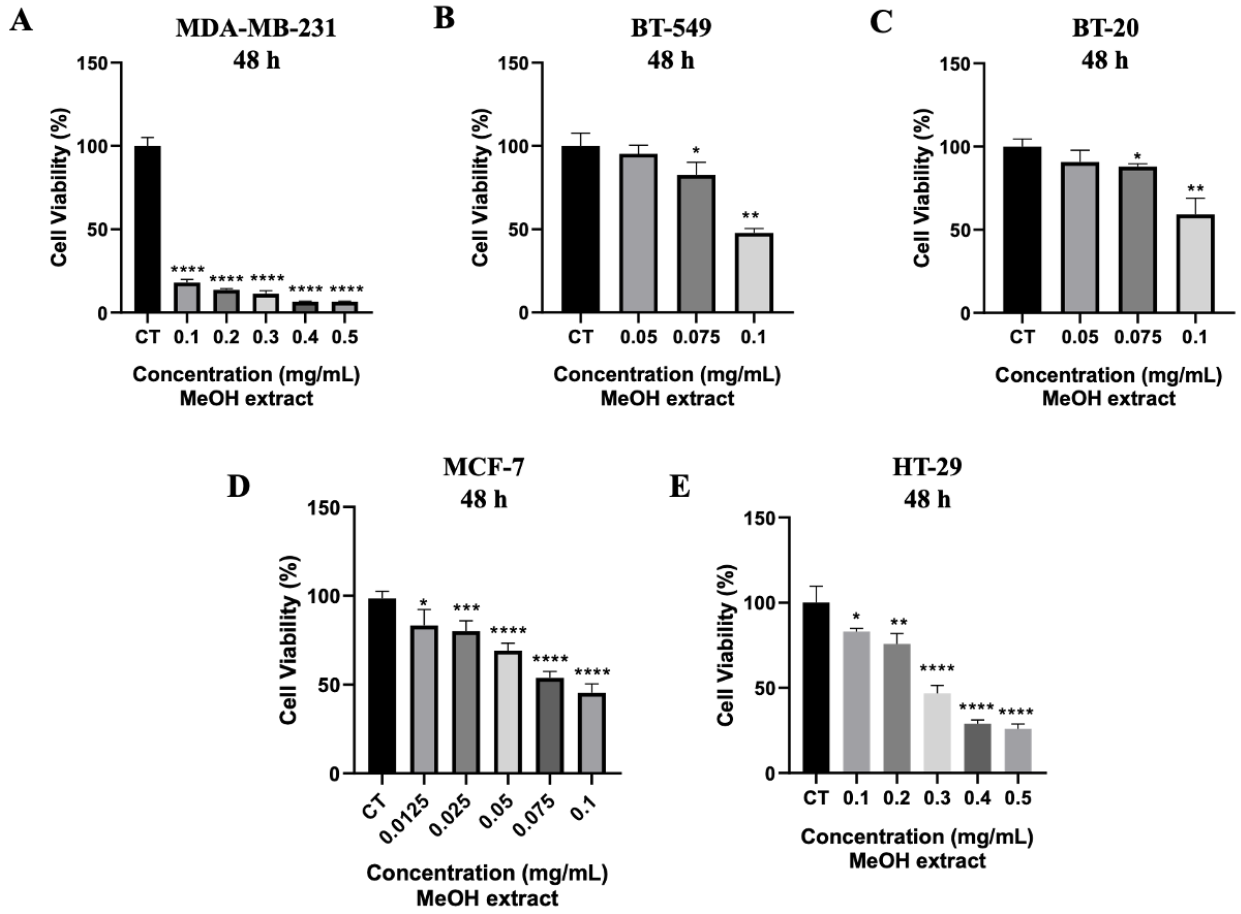

Figure 3. The effect of *P. eryngii* mushroom extracts on breast and colon cancer cell lines. The MeOH extracts showed an antiproliferative effect on A) MDA-MB-231, B) BT-549, C) BT-20, D) MCF-7, and E) HT-29 cell lines after 48 h treatments

According to the reported studies, proteoglycans of TCM mushrooms are associated with the apoptosis process, antiangiogenesis, reversal of drug resistance, and antimetastasis and immune system [27]. In a different study with gold nanoparticles, gold nanoparticles (AuNPs) were isolated from *Commiphora wightii* using an aqueous extract of *Cladosporium* sp., breast cancer cell line (MCF-7), and showed enhanced apoptotic activity [28]. An ethyl acetate fraction of *Lentinula edodes* mushroom induced apoptosis in MCF-7 and MDA-MB-453 cell lines, and the anticancer effect of its isolated bioactive component in MDA-MB-231 and MCF-7 cells was reported [9].

TNBC is identified by the absence of estrogen, progesterone, and Her2 receptors (ER-/PR-/HER2-). The most important feature of these tumors is breast cancer, which is very aggressive and has the lowest patient survival [29]. Therefore, effective and safe treatments against TN breast cancers must be developed due to the lack of targeted therapies. Because of limited response to standard chemotherapeutics and rapid development of resistance, early relapse and lack of therapeutic options are the most important factors that cause high patient mortality rates in TNBC [30]. Colon cancer is the second and third most frequently diagnosed cancer in women and in men, respectively, in the world [31]. Therefore, new sources or therapeutics are needed to discover and develop to fight cancers. These mushroom products from different geographical regions must be deeply evaluated [32].

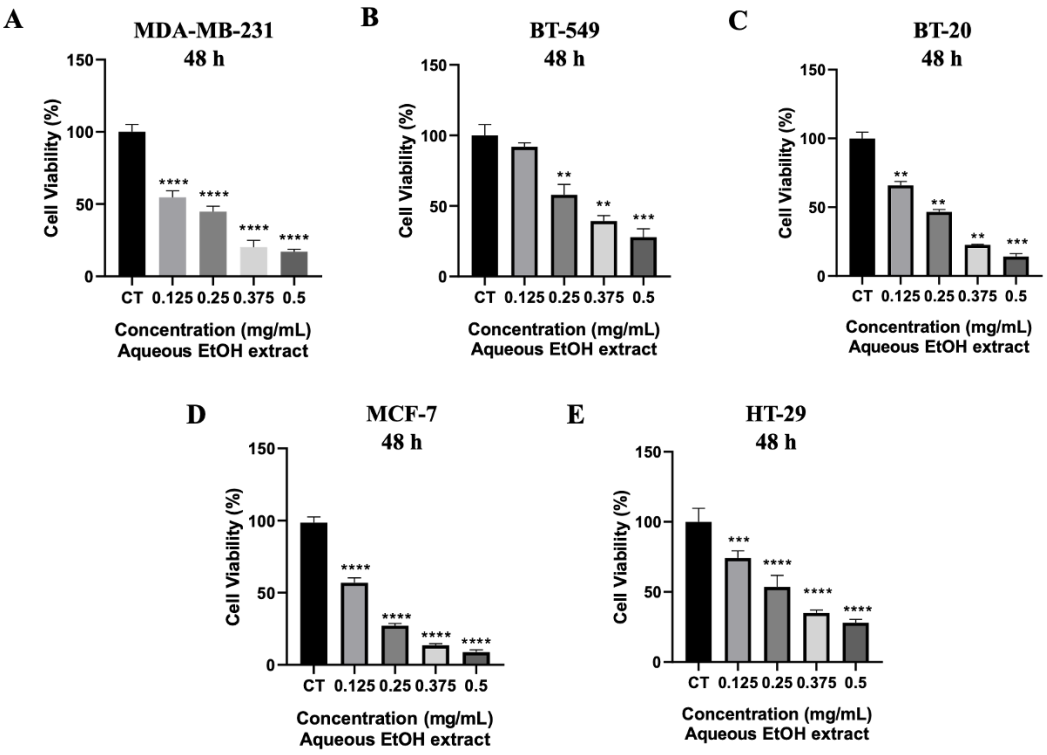

Figure 4. The effect of *P. eryngii* mushroom extracts on breast and colon cancer cell lines. The aqueous EtOH extract showed an antiproliferative effect on A) MDA-MB-231, B) BT-549, C) BT-20, D) MCF-7, and E) HT-29 cell lines after 48 h treatments

3.2. HPLC Analysis

The aqueous EtOH extract was analyzed using high-performance liquid chromatography (HPLC). As given in HPLC chromatograms (Figure 5a-c), the retention times (minutes) of the components were determined at 4.371 (gallic acid), 8.166 (tannic acid), 13.989 (4-hydroxybenzoic acid), 32.463 (salicylic acid), 37.728 (apigenin), 38.033 (4-hydroxy flavone), and 39.092 (chrysin). Gallic acid and 4-hydroxybenzoic acid from the ethanolic and aqueous methanol (80:20) extracts of *P. eryngii* mushroom were identified in reported studies [15, 33]. Furthermore, most phenolics, tocopherols, and carotenoids were determined from ethanolic extracts of *P. eryngii* fruiting bodies by HPLC analysis [15].

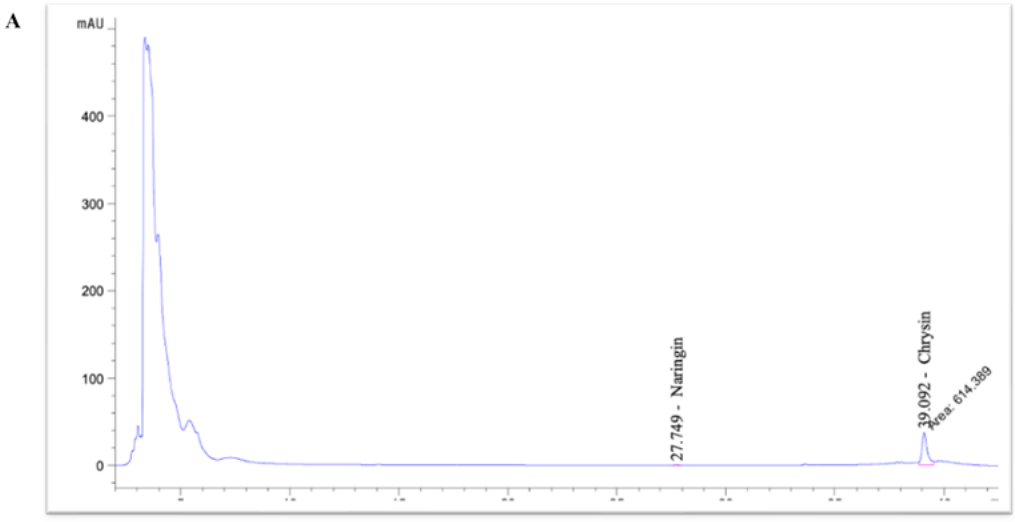

Figure 5. HPLC chromatogram of aqueous EtOH extract of *P. eryngii*

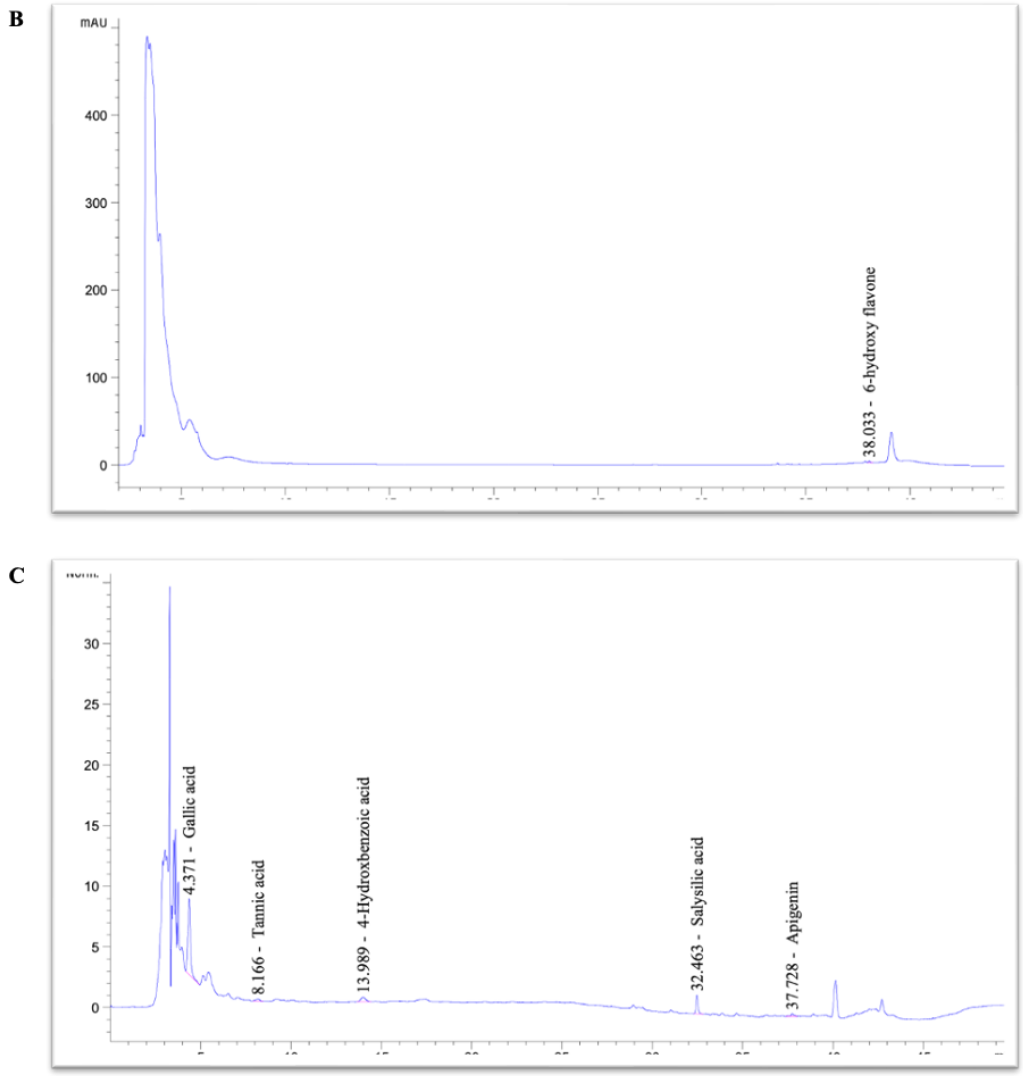

Figure 5. (Continued) HPLC chromatogram of aqueous EtOH extract of *P. eryngii*

4. Conclusion

Edible/medicinal mushrooms are an attractive source for treating human diseases due to their effective bioactive components. Thus, several mushrooms have been used in traditional medicine [10]. Edible *P. eryngii* mushroom collected from Çanakkale was reported for the first time in this study. Therefore, this study focused on investigating the antiproliferative effects of the extracts of *P. eryngii* mushroom against various cancer cell lines. The promising results on cancer cell lines have been obtained following the dose-response treatments. These findings showed that the extracts of *P. eryngii* mushroom were effective at very low concentrations, such as 0.1 mg/mL, on breast cancer cell lines. As indicated in the literature, a bioactive protein and polysaccharides isolated from *P. eryngii* fruiting bodies have been studied on different cancer cell lines and shown significant cytotoxic activity [12, 34]. Consequently, it is thought that there is a need for investigations on edible/medicinal mushroom species with *in vitro* or *in vivo* studies. Thus, these reported studies may lead to the determination of new and effective bioactive components such as polysaccharides, proteins, or phenolics to be used in treating human diseases for further studies.

Author Contributions

All authors analyzed the experiments. All authors contributed to the writing of the manuscript. The first, third, fourth, and fifth authors reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of Interest

All authors declare no conflicts of interest.

Ethical Review and Approval

No approval from the Board of Ethics is required.

Acknowledgment

The cancer cell lines were provided by Erciyes University and Çanakkale Onsekiz Mart University. We would like to thank Ahmet Davutlar for collecting *Pleurotus eryngii* mushroom in Kumkale/Çanakkale and Çanakkale Onsekiz Mart University Experimental Research Application and Research Center (ÇOMÜDAM) for laboratory facilities, and Iğdır University Research Laboratory Practice and Research Center (ALUM) for HPLC analysis. A representative workflow was generated with the help of https://www.biorender.com.

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