

Cytotoxic Activities of Methanol Extract and Compounds of *Porodaedalea pini* Against Colorectal Cancer

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Abstract: *Porodaedalea pini* is a medicinally important mushroom with antioxidant, cytotoxic, immunostimulating, antitumor, antiviral and immunomodulating activities. Therefore, in this study, *P. pini* methanol extract and isolated compounds from the methanol extract were tested for cytotoxic activities against DLD-1 (colorectal cancer) and CCD-18Co (human colon fibroblast cell line) by using Alamar Blue assay. Cytotoxic activity on DLD-1 was decreased in the order of *P. pini* methanol extract > 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**) > pinoresinol (**2**) > ergosta-7,24(28)-dien-3 β -ol (**1**). *P. pini* methanol extract was determined to have the best cytotoxic activity with the lowest IC₅₀ value on DLD-1 (IC₅₀: 25.33±0.29 μ g/mL) and the highest IC₅₀ value on CCD-18Co (434.30±1.45 μ g/mL). Within the scope of the findings, it is thought that *P. pini* mushroom can be used as a new and natural agent in the treatment of colorectal cancer.

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1. INTRODUCTION

Cancer ranks second in deadly diseases after cardiovascular diseases worldwide. Approximately 10% of diagnosed cancers or cancer-related deaths each year consist of colorectal cancer (Dekker et al., 2019). Colorectal cancer is the second most common type of cancer diagnosed in women, while it is third cancer in men. The incidence and mortality in women are about 25% lower than in men. The worldwide incidence of colorectal cancer is estimated to increase to 2.5 million new cases by 2035 (Arnold et al., 2017; Dekker et al., 2019). Among the causes of colorectal cancer, various risk factors including diets are rich in animal fat, in vegetables and fruits, smoking, diabetes and obesity have been identified (Kelly et al., 2012; Perdue et al., 2014). Also, studies have reported that high consumption of red meat increases the risk of colorectal cancer, while adequate physical activity, especially outdoor activities, and the level of vitamin D in the bloodstream reduces the risk of colorectal cancer (Oba et al., 2006; Takachi et al., 2011; Surya et al., 2016). Worldwide, nutrition and diet are the most remarkable factors causing colorectal cancer. The literature studies revealed that many

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natural diet products can be beneficial in the prevention of cancer (Tao et al., 2018). Also, plants, fruits, vegetables, herbal teas, and mushrooms have been reported to have cytotoxic effects on colorectal cancer cells and due to the presence of dietary fibers and phytochemical compounds (Turati et al., 2015; Seidel et al., 2017).

In Chinese medicine, *Phellinus* genus has been reported to be used in the treatment of many diseases such as arthritis of the knee, stomachaches, tumors, inflammation, lymphatic and gastroenteric disorders and to promote longevity in Chinese medicine (Ganeshpurkar et al., 2010; Seephonkai et al., 2011). Bioactive compounds such as sesquiterpenes, triterpenes, steroids, pigments, and polysaccharides, responsible for estrogenic, antiviral, antioxidant, antitumor and anti-estrogenic activity have been purified from *Phellinus* species (Wu et al., 2010; Song et al., 2014; Wang et al., 2014; Pei et al., 2015). *Porodaedalea pini*, a member of *Phellinus* genus and Hymenochaetaceae family, is usually grown under the pine trees. It is known to lower blood sugar, increase immunity, and have anti-cancer properties (Ayer et al., 1996). Antioxidant, antifungal, anticholinesterase, anti-tyrosinase, cytotoxic activities, and inhibition effects on NO production in murine macrophages-RAW 264.7 cells by the compounds isolated from *P. pini* have been revealed by previous studies (Ayer et al., 1996; Wangun & Hertweck, 2007; Jang & Yang, 2011; Deveci et al., 2019^a). In recent years, the discovery of bioactive compounds from natural sources, and obtaining compounds and possible drug candidates with therapeutic properties have attracted more attention. In this context, mushrooms are considered as sources of natural bioactive compounds and are defined as promising therapeutic agents. The study was aimed to evaluate cytotoxic activities against DLD-1 (colorectal cancer) and CCD-18Co (human colon fibroblast cell line) of both *P. pini* methanol extract and ergosta-7,24(28)-dien-3 β -ol, pinoresinol, and 4-(3,4-dihydroxyphenyl)but-3-en-2-one compounds isolated from the methanol extract in our previous study (Deveci et al., 2019^a).

2. MATERIAL and METHODS

2.1. Mushroom Material

Porodaedalea pini (Brot.) Murrill. was collected from the villages of Mentese district of Mugla in November-December 2014 and January 2015 from the area of Muğla, Turkey. The voucher specimen has been stored at the Research and Application Center for Mushrooms, Mugla Sitki Kocman University (Fungarium No AT-2446).

2.2. Spectral Measurements and Chemicals Used

For purification of the compounds, silica gel (Kieselgel 60, 70-230 mesh, Merck) was used for column chromatography; RP-18 F₂₅₄S and silica gel 60 F₂₅₄ plates for thin-layer chromatography (TLC). Separation and isolation of the compounds were performed by using C₁₈ and GS-320 column-linked recycling HPLC (Japan Analytical Industry Co. Ltd.). UV-254-nm light was used for detection of TLC spots and CeSO₄ solution for visualization. Chemical structures of the isolated compounds were elucidated by using FT-IR, 1D-NMR, 2D-NMR spectroscopy techniques. Thermo Scientific one Nicolet IS10 FT-IR spectrometer was used to record the IR spectrum. The NMR spectrum was on Agilent-600-MHz instruments coupled with cooled cryoprobes probe for ¹H- and ¹³C-NMR including HSQC, HMBC, and COSY. A 96-well microplate reader (MultiskanGo, Thermo Scientific Co., MA, USA) was used to analyze cytotoxic activity studies. Cytotoxic activity results were measured and calculated by using GraphPad Prism (GraphPad Software v5.0, USA).

2.3. Extraction and Isolation

For extraction of the aerial parts of *P. pini* (1900 g), powdered mushroom samples were macerated separately and respectively in solvents with increasing polarity: *n*-hexane,

chloroform, acetone, and methanol for 24 h; and four times at room temperature. Solvents were vaporized by using a rotary evaporator. The methanol extract obtained was 49.70 g. The methanol extract was stored at +4 °C for further analysis. As a result of the chromatographic isolation of *P. pini* methanol extract, ergosta-7,24(28)-dien-3 β -ol, pinoresinol, and 4-(3,4-dihydroxyphenyl)but-3-en-2-one were obtained. Details about the isolation and characterization of the compounds can be seen in our previously published research (Deveci et al., 2019^a).

2.4. Cell Viability

CCD-18Co (human colon fibroblast cell line) and DLD-1 (colorectal cancer) were cultivated in EMEM and RPMI-1640 growth mediums (ATCC, Virginia, USA), respectively and incubated with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 2 mM L-glutamine (Sigma, St. Louis, Missouri, USA) in 5% CO₂ at 37 °C and 90-95% humidity.

2.5. Cell Viability Assay

1x10⁴ cells were put into 96-well plate with a growth medium and incubated in 5% CO₂ at 37 °C for 24 h until they attached to the bottom. Different concentrations (between 1 μ g/mL and 1000 μ g/mL) of the methanol extract and isolated compounds of *P. pini* were added to each well. Viability and proliferation of the cells were tested according to the previously described Alamar Blue assay (Karakurt & Adali, 2016). The results were measured at 570 nm and 610 nm by using a 96-well microplate reader. Doxorubicin was used as a positive control. The sigmoidal plot of the inhibition rate (%) versus the log concentration was used to calculate the IC₅₀ values of *P. pini* methanol extract and the isolated compounds.

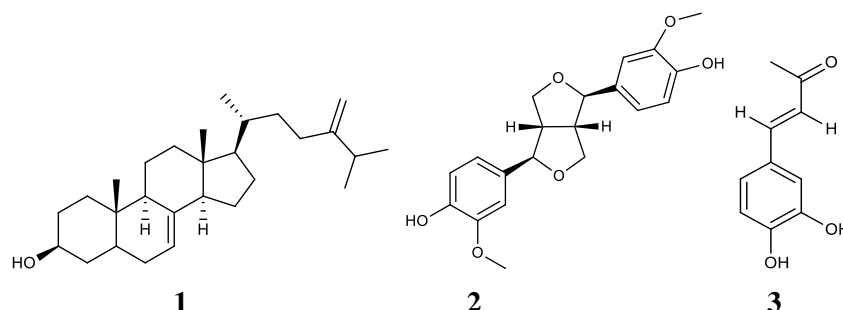
2.6. Statistical Analysis

All data on cytotoxic activity tests were the average of three parallel sample measurements. Data were recorded as mean \pm S.E.M. Significant differences between means were determined by *t*-test, *p* < 0.05 were regarded as significant.

3. RESULTS and DISCUSSION

Ergosta-7,24(28)-dien-3 β -ol (**1**), pinoresinol (**2**), and 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**) were obtained as a result of the isolation of *P. pini* methanol extract. Spectroscopic data of the isolated compounds can be seen in our published previous research (Deveci et al., 2019^a). Figure 1 shows the chemical structures of the compounds isolated from *P. pini* methanol extract.

Figure 1. Chemical structures of the compounds isolated from *P. pini* methanol extract.



Cytotoxic activities against DLD-1 (colorectal cancer) and CCD-18Co (human colon fibroblast cell line) of *P. pini* methanol extract and ergosta-7,24(28)-dien-3 β -ol (**1**), pinoresinol (**2**) and 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**) compounds isolated from the methanol extract were tested by Alamar Blue assay. DLD-1 and CCD-18Co cells were treated with different concentrations of the methanol extract and isolated compounds. Figure 2 represents

the cytotoxic effects of *P. pini* methanol extract and isolated compounds on DLD-1 and CCD-18Co.

Figure 2. Cytotoxic effects of *P. pini* methanol extract and isolated compounds on DLD-1 and CCD-18Co (a) IC₅₀ values on DLD-1 (b) IC₅₀ values on CCD-18Co (c) Heat Map analyses of dose-dependent inhibition against DLD-1 cells. Cell viability decreased from red to pink color (d) Heat Map analyses of dose-dependent inhibition against CCD-18Co. Cell viability decreased from green to pink color.

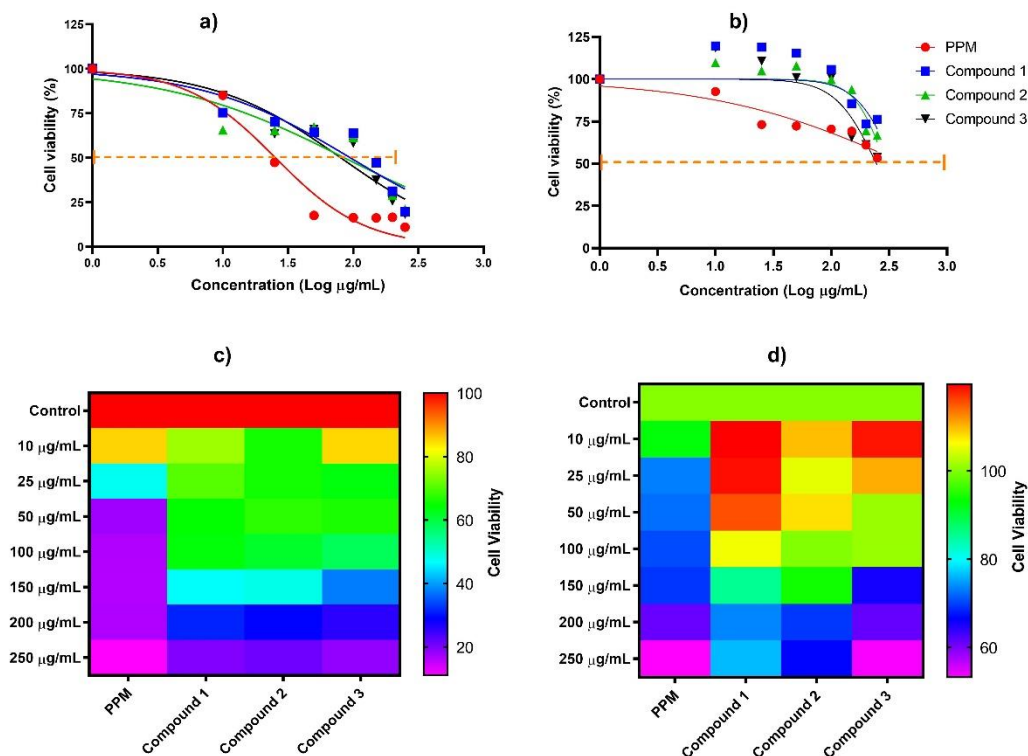


Table 1. Calculated IC₅₀ values of the methanol extract and isolated compounds of *P. pini*^a

	DLD-1 IC ₅₀ (µg/mL)	CCD-18Co IC ₅₀ (µg/mL)
Methanol extract (PPM)	25.33±0.29	434.30±1.45
Ergosta-7,24(28)-dien-3β-ol (1)	95.05±1.25	347.20±0.78
Pinoresinol (2)	85.69±0.87	293.90±0.46
4-(3,4-dihydroxyphenyl)but-3-en-2-one (3)	80.32±0.98	245.20±0.34
Doxorubicin ^b	6.10±0.55	NT ^c

^a: IC₅₀ values represent the means ± SEM of three parallel measurements ($p < 0.05$). ^b: Positive control. ^c: NT: Not tested.

Table 1 shows the calculated IC₅₀ values of *P. pini* methanol extract and isolated compounds. As seen in Figure 2, *P. pini* methanol extract and isolated compounds inhibited the viability of DLD-1 and CCD-18Co in a dose-dependent manner. Cytotoxic activity on DLD-1 was decreased in the order of *P. pini* methanol extract (IC₅₀: 25.33±0.29 µg/mL) > 4-(3,4-dihydroxyphenyl)but-3-en-2-one (3) (IC₅₀: 80.32±0.98 µg/mL) > pinoresinol (2) (IC₅₀: 85.69±0.87 µg/mL) > ergosta-7,24(28)-dien-3β-ol (1) (IC₅₀: 95.05±1.25 µg/mL). Toxicity on CCD-18Co was decreased in the order of *P. pini* methanol extract (IC₅₀: 434.30±1.45 µg/mL) >

ergosta-7,24(28)-dien-3 β -ol (**1**) (IC₅₀: 347.20±0.78 μ g/mL) > pinoresinol (**2**) (IC₅₀: 293.90±0.46 μ g/mL) > 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**) (IC₅₀: 245.20±0.34 μ g/mL) (Table 1).

From ancient times, people have described mushrooms as culinary wonders and valuable in folk medicine. In recent years, mushrooms have witnessed the intense interest of scientists due to their pharmaceutical potentials (Dimitrijevic et al., 2017). Until this time, the medicinal uses of mushrooms have been associated with their nephroprotective, antioxidant, antitumor, hypocholesterolemic, antidiabetic, immunomodulatory, anti-cancer, antiallergic, and antimicrobial properties. Literature studies have shown that *Phellinus*, *Agaricus*, *Pleurotus*, *Clitocybe*, *Ganoderma*, *Antrrodia*, *Cordyceps*, *Trametes*, *Calvatia*, *Xerocomus*, *Flammulina*, *Schizophyllum*, *Suillus*, *Inonotus*, *Funlia*, *Inocybe*, *Lactarius*, *Russula*, *Albatrellus*, and *Fomes* mushroom species are effective against cancer. These mushroom species are defined as new generation biotherapeutics (Patel & Goyal, 2012). It is prominent that not only active isolated compounds but also extracts containing active compounds can be used as alternative agents with fewer side effects in cancer treatment and hence it is important to examine the cytotoxic effects of the mushroom species. According to the results obtained, it was determined that *P. pini* methanol extract has the best cytotoxic activity. In earlier studies, purification and bioactive properties of steroids, phenolics, terpenoids, and polysaccharides from *P. pini* have been reported (Deveci et al., 2019^a; Deveci et al., 2019^b; Hong et al., 2013; Jang & Yang, 2011). Therefore, the higher cytotoxic activity of the methanol extract compared to other isolated compounds can be explained by the synergistic effect of various bioactive compounds contained in it. Among the isolated compounds, phenolic compounds indicated higher cytotoxic properties. It is known that phenolic compounds have anticancer activity associated with their high antioxidant properties or have direct cytotoxic effects on cancer cells (Ivanova et al., 2014). Also, the cytotoxic mechanism of action of phenolic compounds has been elucidated in previous studies as modulating carcinogen metabolism, altering gene expression levels, arresting the cell cycle, inducing apoptosis, and inhibiting various cell proliferation signaling pathways (Huang et al., 2010).

Previously, cytotoxic effects of the extracts, fractions, and pure compounds obtained from different *Phellinus* species on colorectal cancer were determined with a limited number of studies. In the study of Reis et al. (2014), the methanol (GI₅₀: 70±3 μ g/mL) and ethanol (GI₅₀: 61±1 μ g/mL) extracts as also polysaccharides (GI₅₀: 87±4 μ g/mL), glucans (GI₅₀: 202±4 μ g/mL), and triterpenoids (GI₅₀: 65±1 μ g/mL) fractions of *P. linteus* were tested for cytotoxic effects on HCT-15 (colorectal cancer). The hispidine isolated from *P. linteus* has been reported to be a promising new anticancer agent due to inducing both intrinsic and extrinsic apoptotic pathways mediated by ROS in CMT-93 (mouse colorectal cancer) and HCT-116 (human colorectal cancer) cells (Lim, Lee, Park, Kim, & Lim, 2014). He et al. (2015) purified two illudin type sesquiterpenoids, sulphureuine A and phellinuin J from *P. tuberculosis*, tested these compounds for their inhibitory effects on SW480 (colorectal cancer) and no significant inhibitory activity was reported (IC₅₀: >40 mM). Inhibition rates of 24-ethylcholesta-5,22-dien-3 β -ol, ergosterol, 3,4-dihydroxy benzaldehyde, ergosta-7,22-dien-3 β -yl pentadecanoate, baicalein and inoscavin A purified from *P. baumii* on SW620 (colorectal cancer) were reported as ~40, ~30, ~50, ~40, ~90 and ~90%, respectively at 100 μ g/mL concentration. Also, inoscavin A and baicalein were found to have higher cytotoxic activity than 5-fluorouracil that served as a positive control (Zhang et al., 2017). Ethyl acetate extract (IC₅₀: 149.9 μ g/mL), *n*-hexane (IC₅₀: 69.8 μ g/mL), *n*-butanol (IC₅₀: >100 μ g/mL) and ethyl acetate (IC₅₀: 77.8 μ g/mL) fractions and atractylenolide I (~20% cell viability at 100 μ g/mL concentration) from *P. linteus* were investigated for their cytotoxic effects on HT-29 (colorectal cancer) by Jeon et al. (2013). There are studies on the cytotoxic effects of different mushroom species on DLD-1 (colorectal cancer) in the literature. IC₅₀ values of the *n*-hexane, chloroform, and ethyl acetate subfractions of *Tremella fuciformis* were found as 350, 400, and 450 ppm, respectively against DLD-1

(colorectal cancer) (Kim, Chang, Choi, Yoon, & Lee, 2006). In a different study, at 0.2 and 0.5 mg/mL concentrations, *Inonotus obliquus* water extract inhibited 48% and 62% proliferation of HCT-116 and 40% and 60% proliferation of DLD-1 colorectal cancer cells and it was reported that the water extract downregulated the β -catenin and NF- κ B signaling, which exerted anti-inflammatory and antiproliferative activities in colorectal cancer cells (Mishra et al., 2013). Ergosterol peroxide isolated from *Inonotus obliquus* was screened for cytotoxic activity against HCT-116, HT-29, SW620, and DLD-1 colorectal cancer cells at 0, 5, 10, 20 μ g/mL concentrations. When the cell growth of HCT-116, HT-29, SW620 were found nearly ~20% at 20 μ g/mL concentration, the cell growth of DLD-1 was found nearly ~80% at 20 μ g/mL concentration (Kang et al., 2015). Our results are in agreement with the literature studies. This is the first investigation on cytotoxic activities of *P. pini* methanol extract and isolated compounds against DLD-1 and CCD-18Co cells.

4. CONCLUSION

Chemotherapy, cytotoxic drugs, radiotherapy, and surgery are the main methods used in the treatment of colorectal cancer. Among these methods, the most effective method is surgery, and treatment is continued with chemotherapy or radiotherapy since there is a high possibility of recurrence of colorectal cancer after surgery. The negative side effects of chemotherapy compounded by the tendency of colorectal cancer reoccurrence, has made it imperative to search for new, natural, and effective agents with fewer side effects in its treatment. Cytotoxicity of *P. pini* methanol extract and ergosta-7,24(28)-dien-3 β -ol (**1**), pinoresinol (**2**), and 4-(3,4-dihydroxyphenyl)but-3-en-2-one (**3**) isolated compounds were investigated on DLD-1 and CCD-18Co cells for the first time. The methanol extract of *P. pini* displayed significant cytotoxicity on DLD-1. As a result, this study recommends that *P. pini* mushroom, especially the methanol extract, can be used for further research that could lead to the development of new natural remedies in the treatment of colorectal cancer.

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).

Authorship contribution statement

Ebru Deveci: Investigation, Visualization, Software, Formal Analysis, Methodology, Writing-original draft, Supervision. **Gulsen Tel-Cayan:** Investigation, Visualization, Software, Formal Analysis, Methodology, Writing-original draft, Supervision. **Serdar Karakurt:** Resources, Methodology, Validation. **Mehmet Emin Duru:** Resources, Methodology, Formal Analysis, Validation.

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