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Assessment of Cytotoxic Effect of *Porodaedalea pini* Mushroom on Prostate Cancer

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Abstract: *Phellinus* species are in the classification of valuable mushrooms among medicinal mushrooms and have been the source of traditional medicine for centuries. In this study, the hexane (PPH), methanol (PPM) and water (PPW) extracts, fractions and isolated compounds from *Porodaedalea pini* (Çamkavı mantarı) were screened for their cytotoxic effects on PC3 (prostate cancer) and 3T3 (murine fibroblast) cell lines by MTT assay. PPH was found to have moderate cytotoxicity on PC3 cell with the IC_{50} values of 33.84±0.01 µg/mL. The fatty acid profile of PPH was identified by GC-FID (gas chromatography-flame ionization dedector) and GC-MS (gas chromatography-mass spectrometry) and the main fatty acids were recorded as palmitic (41.40±0.03%), linoleic (21.53±0.01%), oleic (12.06±0.01%), and stearic (8.20±0.01%) acids. PPM and PPW were fractioned by using liquid-liquid extractions. Among all fractions, the 2-butanol fraction of the methanol extract (PPM-B) indicated the best cytotoxicity on PC3 and 3T3 cell lines. Also, among the isolated compounds (dioctyl phthalate (**1**), ergosterol peroxide (**2**) and pinoresinol (**3**)) from PPM, ergosterol peroxide (**2**) was found as moderate cytotoxic on PC3 with the IC_{50} value of 95.47±1.01 µg/mL. This study, in which the effect of *P. pini* on PC3 cell was examined for the first time, proved its medicinal importance by revealing the cytotoxic properties of the species.

Key words: *Porodaedalea pini,* Prostate cancer, Extract, Fatty acid, Isolation

Porodaedalea pini Mantarının Prostat Kanseri Üzerindeki Sitotoksik Etkisinin Değerlendirilmesi

Öz: *Phellinus* türleri şifalı mantarlar arasındaki değerli mantarlar sınıfında yer almaktadır ve yüzyıllardır geleneksel tıbbın kaynağı olmuştur. Bu çalışmada, *Porodaedalea pini* (Çamkavı mantarı)'den elde edilen hekzan (PPH), metanol (PPM) ve su (PPW) ekstrelerinin, fraksiyonlarının ve izole edilmiş bileşiklerin PC3 (prostat kanseri) ve 3T3 (fare fibroblast) hücre hatları üzerindeki sitotoksik etkileri MTT testi kullanılarak taranmıştır. PPH'ın 33.84±0.01 µg/mL olan IC_{50} değeri ile PC3 üzerinde orta derecede sitotoksisiteye sahip olduğu bulunmuştur. PPH'ın yağ asidi profili GC-FID (gaz kromatografisi-alev iyonizasyon dedektörü) ve GC-MS (gaz kromatografisi-kütle spektrometresi) ile belirlenmiş ve majör yağ asitleri palmitik (%41.40±0.03), linoleik (%21.53±0.01), oleik (%12.06±0.01) ve stearik (%8.20±0.01) asit olarak kaydedilmiştir. PPM ve PPW ekstreleri, sıvı-sıvı ekstraksiyon tekniği kullanılarak fraksiyonlandırılmıştır. Tüm fraksiyonlar arasında metanol ekstresinden elde edilen 2-bütanol fraksiyonu (PPM-B) PC3 ve 3T3 hücre hatlarında en iyi sitotoksisiteyi göstermiştir. Ayrıca, PPM'den izole edilen bileşiklerden (dioktil ftalat (**1**), ergosterol peroksit (**2**) ve pinoresinol (**3**)), ergosterol peroksit (**2**) 95.47±1.01 µg/mL olan IC_{50} değeri ile PC3 hücre hattında orta derecede sitotoksik olarak bulunmuştur. *P. pini*'nin prostat kanseri üzerindeki etkisinin ilk kez incelendiği bu çalışmada, türün sitotoksik özellikleri ortaya çıkartılarak tıbbi önemi kanıtlanmıştır.

Anahtar Kelimeler: *Porodaedalea pini,* Prostat kanseri, Ekstre, Yağ asidi, İzolasyon

Introduction

Cancer still maintains its importance as the leading cause of death worldwide. Factors such as morbidity, poor prognosis, recurrence and survival rate of the disease are encountered as challenges in traditional non-surgical treatment procedures such as radiotherapy and chemotherapy (Zhang et al., 2020). Survival in cancer patients, especially in advanced phases, remains at low levels in proportion to factors such as high toxicity, drug resistance and other long-term side effects. Therefore, the tendency of scientists to search for more effective approaches and discover novel anticancer drugs in this direction has gained momentum (Tilaoui et al., 2021).

Prostate cancer is the second most common cancer in men, especially affecting older men: more than 80% of cases are diagnosed after age 65 (Daniyal et al., 2014). Studies based on the number of new cases rank prostate cancer as the sixth most prevalent cancer in the world. It is registered as the most common type of cancer in men in North America, parts of Africa and Europe, and as the second leading cause of cancer death in men in the United States (Grönberg, 2003; Hsing and Chokkalingam, 2006). However, only 10% of men with prostate cancer die from the disease. This is the paradox of prostate cancer: at autopsy of men aged 70-79, prostate cancer is 39%, increasing to 43% by age 80. Identified risk probabilities of prostate cancer are race, age, and positive family history. Other risk factors such as hormones, occupation, dietary factors, obesity, physical activity, sexual activity, smoking, genetic susceptibility and vasectomy have also been connected with prostate cancer risk, but their role in the aetiology remains unclear. It is estimated that 42% of prostate cancer risk is due to genetic influences, including individual and combined effects of rare, highly penetrant genes, more commonly poorly penetrating genes, and genes acting in concert with each other. Prostate cancer pathogenesis probably involves interaction between environmental and genetic factors (Hsing and Chokkalingam, 2006; Rawla, 2019).

Nature has been a significant source of inspiration for medicine since ancient times. Nature produces a wide variety of biologically active constituents with amazing therapeutic potential as well as resources related to cancer treatment (Majolo et al., 2019). The fact that natural products dominate a wide range of traditional and folk medicine applications in the treatment of many diseases and are the source of several compounds commonly used in cancer chemotherapy has generally allowed them to lead to potential new compounds. First-generation chemotherapeutics are more active against rapidly proliferating cancer cells, as well as act on therapeutic interactions that are not specific to cancer. This treatment causes a variety of harmful side effects that can have consequences in anticancer chemotherapy, from overdose to patient death. Newer methods for cancer treatment include targeted therapies that are specific to the properties of cancer and cause harmless or minimal damage to healthy tissues (Hanahan and Weinberg, 2011). Therefore, the search for compounds that can act selectively on cancer cells is a top priority issue in this field. It is therefore a very important responsibility to uncover the treasury of natural products for the discovery of a variety of highly specific agents to make modern oncology more powerful.

Cancer mycotherapy is documented as a promising scientific arena that studies anticancer substances derived from mushrooms. Around the world, mushrooms have become a fundamental part of traditional medicine, thanks to their valuable bioactive properties (Xu et al., 2012). The concept of mushroom therapy has been officially introduced in Traditional Chinese Medicine, as it is recognized as the most effective natural remedies for various kinds of cancer (Hao and Jiang, 2015). Since medicinal mushrooms are producers of hundreds of compounds (heteropolysaccharides, phenolic acids, α-glucans, proteins, β-glucans, fatty acids, complexes of polysaccharides with proteins, terpenoids, nucleoside antagonists, sesquiterpenes, lanostanoids, and sterols), they can synergistically affect more than one cancer-related pathway during treatment. Therefore, investigation of complex anticancer effects contributed by the molecular combinations of extracts and fractions, as well as pure mushroom-derived compounds, is among the important studies focused on (Popovic et al., 2013).

Phellinus species belong to the family of medicinal mushrooms and have been recognized for centuries in traditional medicine of the Far East. It has been used as an effective remedy for bleeding, stomach and intestinal ailments, and diarrhea. In mycochemical studies, it was emphasized that this species showed biological properties such as mainly antiviral, antioxidant, antiangiogenic, and anticancer associated with the presence of steroids, polysaccharides, terpenoids, and phenolic compounds (Sulkowska-Ziaja et al., 2021). *Porodaedalea pini* (Çamkavı mantarı) mushroom, a member of the *Phellinus* family, is a species that usually grows on pine trees and is widely distributed in the northern hemisphere (Sesli et al., 2020). In the studies, it has been reported that the extracts (methanol, ethanol, water and polysaccharide extracts) and pure compounds (steroids, terpenes and phenolic compounds) obtained from *P. pini* have antioxidant, anti-cancer, anti-viral and anti-inflammatory properties (Deveci et al., 2019a; Deveci et al., 2021; Hong et al., 2012; Lee et al., 2010).

The proportion of studies focusing on mushrooms has increased exponentially in recent years (Blagodatski et al., 2018). This study adds the first information to the literature by investigating the cytotoxic effects of *Porodaedalea pini* on PC3 (prostate cancer) and 3T3 (murine fibroblast) cell lines. The cytotoxic effects of the extracts, fractions and isolated compounds were elucidated with the chemical profile of the most active extract.

Material and Metod

Mushroom Material

Porodaedalea pini (Brot.) Murrill. (Çamkavı mantarı) was collected from Muğla, Türkiye in November-December 2014 and January 2015. The voucher specimen was stored with Fungarium No: AT-2446 in the Research and Application Center for Mushrooms, Mugla Sitki Kocman University.

Extraction and isolation

The powdered aerial parts of *P. pini* were macerated with two different solvents (*n*-hexane and methanol) at room temperature, respectively. Solvents were removed by using a rotary evaporator to get hexane (PPH) and methanol (PPM) extracts. Then the mushroom residue was extracted with water at 80°C for one day and lyophilized to obtain the water extract (PPW).

A part of the methanol extract (PPM) was dissolved in water and liquid-liquid extractions were performed with ethyl acetate and 2-butanol saturated with water, respectively. Thus, ethyl acetate fraction (PPM-EA), 2-butanol fraction (PPM-B) and water fractions (PPM-W) were obtained from the methanol extract. Similarly, the water extract (PPW) was dissolved in water again and a liquid-liquid extraction was performed with 2-butanol saturated with water. Thus, 2-butanol (PPW-B) and water (PPW-W) fractions were obtained from the water extract. All extracts and fractions were stored at +4°C until analysis.

The methanol extract (PPM) was chromatographed by using silica gel column with hexane:$CHCl_3$, $CHCl_3$:acetone, acetone:methanol and methanol to afford twenty-three fractions. 4th fraction (PPM4) was re-fractionated by using silica gel column with the gradient solvent system of hexane:ethyl acetate to give fifteen sub-fractions. Sub-fraction 4th (PPM4-4) was subjected to the silica gel column chromatography using hexane:ethyl acetate (95:5) solvent system to yield compound **1**. Compound **2** was seperated from sub-fraction 9th (PPM4-9) by silica gel column chromatography using hexane:ethyl acetate (85:15) solvent system. Fraction 13th (PPM13) was chromatographed by using silica gel column with gradient solvent system of hexane:ethyl acetate to obtain four sub-fractions. Compound **3** was purified by recycling HPLC (C_{18} column and methanol:water (70:30)). Compounds were identified as dioctyl phthalate (**1**), ergosterol peroxide (**2**) and pinoresinol (**3**) based on their spectroscopic data (IR, ^{1}H-NMR, ^{13}C-NMR and MS) which were in agreement with those published previously. Details about the isolation and characterization of the compounds can be found in our previous published research (Deveci et al., 2019b).

Fatty acid profile

Fatty acids of the hexane extract of *P. pini* (PPH) were investigated by using the transesterification procedure and the analysis was performed by GC-FID and GC-MS as previously reported by Çayan et al. (2020).

A flame ionization detector (FID) and a DB-1 fused silica capillary non-polar column (30m x 0.25 id., film thickness 0.25 µm) were used for GC (Shimadzu GC17 AAF, V3, 230 V series (Japan)) analyses of the methyl derivatives of fatty acids. Injector and detector temperatures were 250 and 270°C, respectively, carrier gas was He at a flow rate of 1.4 mL/min; sample size, 1.0 µL; split ratio, 50:1. The initial oven temperature was held at 100°C for 5 min, then increased up to 238°C with 3°C/min increments and held at this temperature for 9 min. The relative percentages of the fatty acid methyl derivatives were determined with GC solution computer program. An ion trap mass spectrometer (MS) and a DB-1 MS fused silica non-polar capillary column (30 m x 0.25 mm ID, film thickness 0.25 µm) were used for the GC-MS (Varian Saturn 2100 (USA)) analyses of the methyl derivatives of fatty acids. For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Carrier gas was helium (15 psi) at a flow rate of 1.3 mL/min. Injector and MS transfer line temperatures were set at 220°C and 290°C, respectively. The oven temperature was held at 100°C for 5 min, then increased up to 238°C with 3°C/min increments and held at this temperature for 9 min. Diluted samples (1/25, w/v, in *n*-hexane) of 0.2 µL were injected manually in the split mode. Split ratio was 50:1. EI-MS were taken at 70 eV ionization energy. Mass range was from m/z 28 to 650 amu. Scan time 0.5 sec with 0.1 inters scan delays. The library search was carried out using NIST and Wiley 2005 (Gas Chromatography-Mass Spectrometry) GC-MS libraries. FAME (fatty acid methyl ester) mixture (Supelco™ 37, Catalog no: 47885-U) were identified by comparing their retention times with those of the pure FAMEs standards.

The fatty acid profile was determined by three parallel measurements. Results were given as relative percentage (%) of each fatty acid.

Determination of the cytotoxicity

RPMI1640 media containing 10% FBS (fetal bovine serum) was used for the culture of PC3 (prostate cancer) cells and Dulbecco's Modified Eagle Medium containing 5% FBS (fetal bovine serum), penicillin (100 IU/mL) and streptomycin (100 µg/mL) for 3T3 (murine fibroblast cell line). Both cell cultures were incubated at 37°C in a humidified in the atmosphere of 5% CO_2 After adding 5x10^4 cells with the respective growth medium to the 96-well plate, they were left to incubation in 5% CO_2 at 37°C for 24 h until adhered to the bottom. Sample solution including the extracts, fractions and isolated compounds of different concentration (1-200 µg/mL) was added to each well. Viability and proliferation of the cells were tested according to the previously described MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Deveci et al., 2019c). The results were read at 540 nm. Doxorubicin was used as standard for

PC3 and cycloheximide for 3T3 cell lines and the cell viability values were expressed as 50% inhibition concentration (IC_{50}).

Statistical analysis

All results were the average of three parallel sample measurements and given as the mean ± S.D. (standard error). Student's *t* test was used to analyse significant differences and *p* values <0.05 were accepted as significant.

Results

Fatty acid profile

Fatty acid profile of *P. pini* was investigated by GC-FID and GC-MS and all identified fatty acids were listed in Table 1. GC chromatogram of *P. pini* hexane extract (PPH) was presented in Figure 1. The dominant fatty acids were detected as palmitic (41.40±0.03%), linoleic (21.53±0.01%), oleic (12.06±0.01%), and stearic (8.20±0.01%) acids among the eleven identified fatty acids. The total fatty acid contents for SFAs, MUFAs, and PUFAs were calculated as 54.98, 18.49, 26.24%, respectively.

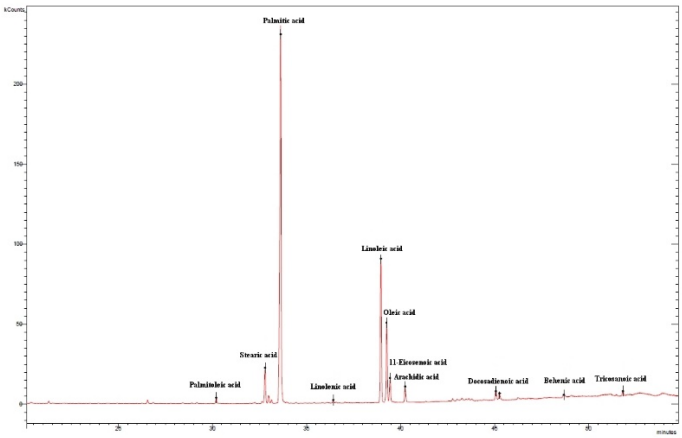

Figure 1. GC chromatogram of *P. pini* hexane extract (PPH).

Cytotoxic activity

The extracts, fractions and isolated compounds obtained from *P. pini* were investigated against PC3 and 3T3 cell lines using MTT assay. Firstly, the cytotoxic activities of the hexane (PPH), methanol (PPM) and water extracts (PPW) obtained from *P. pini* and the fractions obtained from these extracts using liquid-liquid extraction on PC3 and 3T3 cells were determined. The results were summarized in Table 2. The hexane extract (PPH) showed the best cytotoxicity on PC3 (IC_{50}: 33.84±0.01 µg/mL), while methanol extract (PPM) exhibited moderate cytotoxic activity on 3T3 (IC_{50}: 38.05±0.14 µg/mL). The National Cancer Institute and Geran protocol described the cytotoxicity of the extracts as high ($IC_{50} \leq 20$ µg/mL), moderate (IC_{50}: 21-200 µg/mL), weak (IC_{50}: 201-500 µg/mL), and not ($IC_{50} \geq 501$ µg/mL) cytotoxic (Nguyen et al., 2020).

Table 1. The fatty acid profile of *P. pini*[a]

Fatty Acids	%
Palmitic acid (C16:0)	41.40±0.03
Palmitoleic acid (C16:1 ω-7)	3.05±0.02
Stearic acid (C18:0)	8.20±0.01
Oleic acid (C18:1 ω-9)	12.06±0.01
Linoleic acid (C18:2 ω-6)	21.53±0.01
Linolenic acid (C18:3 ω-3)	1.99±0.01
Arachidic acid (C20:0)	2.47±0.02
11-Eicosenoic acid (C20:1 ω-9)	3.38±0.01
Behenic acid (C22:0)	1.06±0.01
Docosadienoic acid (C22:2 ω-6)	2.72±0.03
Tricosanoic acid (C23:0)	1.85±0.02
Total saturated fatty acids (SFAs)	54.98
Total monounsaturated fatty acids (MUFAs)	18.49
Total polyunsaturated fatty acids (PUFAs)	26.24
Total unsaturated fatty acids (UFAs)	44.73
Undetected fatty acids	0.29

[a] Fatty acid profile represent the means ±S.D. of three parallel sample measurements (p<0.05).

According to this comparison, the methanol extract (PPM) was moderately cytotoxic against PC3 with IC_{50} value of 88.77±0.78 µg/mL, while water extract (PPW) was determined as weak active. Among the all fractions, PPM-B was recorded as a moderate cytotoxic fraction against PC3. Secondly, a portion of the methanol extract (PPM) was purified as a result of a combination of different chromatographic techniques as described in our previous study (Deveci et al., 2019b). The chemical structures of the compounds isolated were presented in Figure 2. Among the isolated compounds, only ergosterol peroxide (**2**) (IC_{50}: 95.47±1.01 µg/mL) showed moderate cytotoxicity on PC3 (Table 2).

Discussions

Fatty acids cause changes in metabolism by affecting gene expression, hormonal responses and the properties of cells, and also take part in the production processes of biologically active compounds. On these occasions, fatty acids act on physiological functions, health and disease risk (Calder, 2015). It has been proven that fatty acids (especially ω-3 and ω-6) enhance and even protect the effect of medical treatment for important diseases such as diabetes, cardiovascular diseases and cancer (Çakmakçı and Tahmas-Kahyaoğlu, 2012). GC-FID is a traditional technique used in the analysis of fatty acids due to its high accuracy, sensitivity and suitability. GC-MS is used for the determination of fatty acids for more precise quantitative determination. Incorporating the high resolution of GC and the high sensitivity of MS to separate and identify complex components, this technique is an effective tool for qualitative and quantitative analysis of fatty acids. In this context, it is recommended in the literature to use both techniques together for the identification of fatty acids (Chiu and Kuo, 2020; Wu et al., 2017).

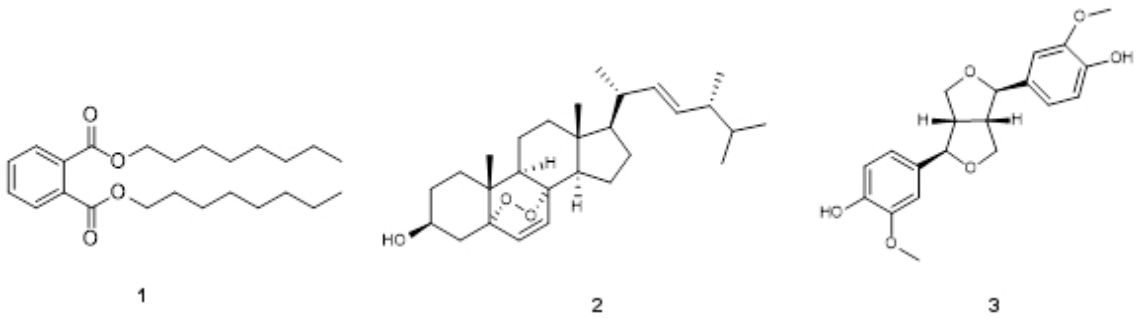

Figure 2. Chemical structures of the compounds isolated from *P. pini* methanol extract (PPM). 1: dioctyl phthalate, 2: ergosterol peroxide, 3: pinoresinol

Many extensive *in vitro* and *in vivo* studies have examined the association and risk of fatty acids with various types of cancer species such as breast, prostate, and colorectal cancer (Chen and Huang, 2019).In this study, fatty acid profile of *P. pini* hexane extract (PPH) was investigated due to have the highest cytotoxicity on PC3 cell line and the main fatty acids were detected as palmitic (41.40±0.03%), linoleic (21.53±0.01%), oleic (12.06±0.01%), and stearic (8.20±0.01%) acids by GC-FID and GC-MS. According to the study of Reis et al. (2014) the main fatty acids were consisted of palmitic (26.8±0.2%), linoleic (25.3±0.2%), oleic (13.5±0.1%) and stearic (12.3±0.1%) acids in *P. linteus*. The most abundant fatty acids of *P. pini* were described as linoleic (41.9%), palmitic (19.2%) and oleic (14.4%) acids (Olennikov et al., 2014). In our previous study, the main fatty acids of *P. igniarius* were linoleic (48.27±1.19%), palmitic (28.09±1.02%) and stearic (7.42±0.11%) acids (Çayan et al., 2020). Palmitoleic (0.00461-0.10032 mg/g), linoleic (0.1629-1.6483 mg/g), oleic (0.01644-0.10889 mg/g), hexadecanoic (0.3952-1.2403 mg/g), and stearic (0.1464-0.3821 mg/g) acids were identified in six *Phellinus* species (*P. linteus, P. baumii, P. pomaceus, P. pini, P. robustus, Phellinus sp*.) (Deng et al., 2011).

Treatment of prostate cancer is performed using surgery, radiotherapy, hormonal therapy, neoadjuvant hormone therapy and androgen withdrawal therapy approaches (Sumanasuriya and De Bono, 2018). The method to be used for the treatment of prostate cancer varies according to the stage of cancer. For this reason, patients in whom the cancer process progresses become resistant to treatment and treatment options become difficult. Antiandrogen manipulation, estrogen therapy, use of chemotherapeutic drugs such as gemcitabine, doxorubicin and paclitaxel are common in advanced prostate cancer patients (Kroon et al, 2014). However, the acquired drug resistance of these treatment methods and the inadequacy of the dose to be used or the dose-dependent side effects are not sufficient for prostate treatment, but often only increase the survival rate of the patient. For this purpose, studies on the characterization of new strategies, comprising of the use of natural compounds in combination therapies, have gained momentum in the light of the results focusing on the toxic effects of chemotherapy in general. The main goal of incorporating natural compounds into cancer chemotherapies is to expand the therapeutic window of chemotherapeutic drugs and reduce the formation of chemotherapy resistance (Lin et al., 2020).

Table 2. Cytotoxic activity of the extracts, fractions and isolated compounds from *P. pini*[a]

		PC3	3T3
Extracts	PPH	33.84±0.01	40.26±0.45
	PPM	88.77±0.78	38.05±0.14
	PPW	>200	>200
Fractions	PPM-EA	>200	95.65±3.36
	PPM-B	95.01±1.08	>200
	PPM-W	>200	52.05±0.18
	PPW-B	>200	>200
	PPW-W	>200	>200
Isolated compounds	Dioctyl phthalate (**1**)	>200	NT[b]
	Ergosterol peroxide (**2**)	95.47±1.01	NT[b]
	Pinoresinol (**3**)	>200	NT[b]
Standards	Doxorubicin	1.38±0.16	NT[b]
	Cycloheximide	NT[b]	0.07±0.12

[a] IC_{50} values (µg/mL) represent the mean ±S.D. of three parallel sample measurements (p<0.05).
[b] NT: not tested.

PC3 cell, which is considered a classical prostate cancer cell line, is used as an androgen-independent prostate cancer model. This cell line has a high metastatic potential compared to other prostate cancer cell line models (Kamalidehghan et al., 2018). Therefore, the extracts, fractions and isolated compounds obtained from *P. pini* were investigated against PC3 and 3T3 cell lines by MTT assay.

The cytotoxic activities of the hexane (PPH), methanol (PPM) and water extracts (PPW) obtained from *P. pini* and the fractions obtained from methanol and water extracts using liquid-liquid extraction on PC3 and 3T3 cells were determined. The hexane extract (PPH) containing palmitic acid as the main fatty acid showed the best cytotoxicity on the PC3 cell line. *In vitro* and *in vivo* studies have shown that palmitic acid inhibits growth in prostate cancer cells and causes inhibition of cell metastasis by blocking key molecules of the PI3K/Akt pathway (Zhu et al., 2021). The methanol extract (PPM) was moderately cytotoxic against PC3 while water extract (PPW) was determined as weak active. Among all fractions, the 2-butanol fraction of the methanol extract (PPM-B) was recorded as a moderate cytotoxic fraction against PC3. The fractionation with ethyl acetate and butanol derivatives, respectively, is a frequently used separation technique in bioactivity determination studies. The strong anti-angiogenic effect of the butanolic fraction obtained from *P. linteus* mushroom has been proven (Kim et al., 2004). We previously reported the organic acid and phenolic compounds of *P. pini* as *p*-hydroxybenzoic acid (32.40 μg/g), *p*-coumaric acid (5.46 μg/g), caffeic acid (4.07 μg/g), ellagic acid (0.32 μg/g), fumaric acid (0.14 μg/g), vanillin (0.07 μg/g), and coumarin (0.01 μg/g) by using HPLC-DAD (Deveci et al., 2019b). The cytotoxic effects of the extracts may be due to the combined effects of fatty acids and phenolic and organic acid compounds.

Dioctyl phthalate (**1**), ergosterol peroxide (**2**) and pinoresinol (**3**) purified from the methanol extract (PPM) were investigated for the cytotoxicity on the PC3 cell line. Among the isolated compounds, only ergosterol peroxide (**2**) showed cytotoxicity on PC3. It has been proven that sterols promote the growth and apoptosis of cancer cells through the activation of caspase enzymes and inhibit the development of various cancers. The increased activity of caspase enzymes has been attributed to the fact that sterols cause changes in membrane structure and functions as a result of binding to the cell membrane, and this change increases the caspase enzyme activities of proteins included in extracellular and intracellular signal transmission pathways. These two combined pieces of evidence support the anti-cancer effects of sterols, arguing that their inclusion in the diet is an important strategy in the prevention/treatment of cancer (Woyengo et al., 2009).

In a previous study, the inhibition rates of the isolated compounds ergosterol, baicalein, ergosta-7,22-dien-3*β*-yl, 24-ethylcholesta-5,22-dien-3*β*-ol pentadecanoate, 3,4-dihydroxy benzaldehyde, and inoscavinA from *P. baumii* on LNCaP (prostate cancer) were found as ~10, 10, 80, 85, 85, 20%, respectively at 100 μg/mL concentration (Zhang et al., 2017). In a different study, the mechanism of action of ProstaCaid™, which consists of 33 different comprehensive polyherbal and nutritional preparations, including mycelium of *P. linteus* mushroom, is used as a nutritional supplement in prostate cancer patients, on prostate cancer was revealed. PC3 cells were inhibited in a dose- and time-dependent manner with IC_{50} values of 56.0, 45.6 and 39.0 μg/mL for 24, 48 and 72 h, respectively and the proliferation was inhibited through modulation of the genes expression (Jiang et al., 2011). It was also reported that *P. linteus* blocked the growth of prostate cancer cells and induced apoptosis *in vitro* (Guo et al., 2007; Tsuji et al., 2010; Zhu et al., 2007). The sulforhodamine B-based assay was used to test the cytotoxic activity of ergosterol peroxide (isolated from *Hericium novae-zealandiae* mushroom) against DU145 (IC_{50}: 21±3 μM), PC3 (IC_{50}: 42±3 μM), LNCaP (IC_{50}: 15±2 μM) prostate cancer cell lines (Chen et al., 2019). The resazurin reduction test was used to determine the cytotoxicity of ergosterol peroxide (IC_{50}: 38.19±1.67 μM) isolated from *Inonotus obliquus* on PC3 by Ma et al. (2013). The cell growth values of pinoresinol on PC3 (143±55%) and LNCaP (55±9%) prostate cancer cell lines at 100 μM concentration were reported by using MTT assay (Sepporta et al., 2013). Chin et al. (2006) calculated the ED_{50} value of pinoresinol as 0.5 μg/mL against LNCaP (prostate cancer) by the sulforhodamine B-based assay. The differences between the findings can be explained by the effect of a wide variety of variables as follows: method differences, researcher's knowledge and experience, cell type used, ambient temperature, test reagent content and media composition (Tokur and Aksoy, 2017).

This study presents a detailed assessment in terms of comparing the effects of *P. pini* mushroom extracts, fractions and isolated compounds on PC3 and 3T3 cell lines for the first time. The strong cytotoxicity of the hexane extract against PC3 may be related to the contents of palmitic, linoleic, oleic and stearic acids detected by GC-FID and GC-MS. These results demonstrated that it is possible to evaluate *P. pini* (especially hexane extract) as a new agent in prostate cancer. However, it is still necessary to purify cytotoxic compounds with advanced chromatographic techniques and to complete the findings with *in vivo* studies.

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