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ORIGINAL PAPER

Screening of mushrooms bioactivity: piceatannol was identified as a bioactive ingredient in the order Cantharellales

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Abstract
Wild edible mushroom species are appreciated for consumption due to their high nutritional value. The aim of the present study was to examine in vitro beneficial bioactivity of mushroom extracts and to investigate the molecular identity of the active ingredients. In this regard, methanol extracts of 29 different wild edible mushroom species, that are traditionally consumed by residents in the National Park of North Pindos in North-Western Greece, were examined for antioxidant, antiproliferative, cytotoxic, and pro-apoptotic activities towards a human lung adenocarcinoma cell line A549 by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and by flow cytometry. Certain mushroom species exhibited high antioxidant activity, which was related to their high content in total phenols and flavonoids. Methanol extracts of *Cantharellus cibarius, Cantharellus cinereus, Craterellus cornucopioides* and *Hydnum repandum*, which belong to the order Cantharellales, exhibited high cytotoxicity and induced apoptosis–necrosis to A549 cells. High Performance Liquid Chromatography (HPLC) coupled with Mass Spectrometry analysis revealed as an active ingredient piceatannol ((E)-4-[2-(3,5-dihydroxyphenyl)ethenyl]1,2-benzenediol-3,3′,4,5′-tetrahydroxy-*trans*-stilbene). Piceatannol, according to our best knowledge, is identified for the first time in wild edible mushrooms. Experiments with authentic piceatannol confirmed the potent antiproliferative activity of this compound. Tested mushrooms are promising sources of bioactive compounds.

Keywords Mushrooms · Cantharellales · Antiproliferative activity · Cytotoxic and pro-apoptotic activities · Piceatannol

Introduction

Although mushrooms constitute a kingdom containing more than 140,000 species [1], only a few of them (approximately 10%) are known. Among them, about 2000 species are considered to be edible. In Greece, there have been recorded about 3500 mycetes, 1800 from which are mushrooms [2].

Approximately 1200 mycetes are used in 85 different countries for nutritious, pharmaceutical and medicinal purposes. Their high content in protein, low total fat level and high percentage of polyunsaturated fatty acids, make them exceptionally attractive in low calorie diets [3]. Furthermore, mushrooms are rich in functional food ingredients, such as dietary fibers, peptides, lectins, phenolics, terpenes, alkaloids, vitamins, and minerals [4]. Mushroom species are considered as important sources of pharmaceutical products because they contain compounds with antibacterial, antimycotic, and antioxidant activity [5–8]. Results from several investigations suggest that certain higher hetero and homobasidiomycetes might possess anticancer activity [1, 9, 10]. Researchers have pointed out that polysaccharides and especially β-glucans might be involved in this activity; however, compounds with antitumor activity have not been screened and clearly defined.

Piceatannol ((E)-4-[2-(3,5-dihydroxyphenyl)ethenyl]1,2-benzenediol-3,3′,4,5′-tetrahydroxy-*trans*-stilbene) belongs to natural stilbenes and was first isolated in 1956 from *Vouacapoua* species [11]. It is present in small quantities in *Euphorbia lagascae* [12], in grapes [13], in *Vaccinium berries* [14] and in *Arachis hypogaea* [15]. Several studies have shown that piceatannol possesses significant antioxidant, anticancer, and anti-inflammatory activity [16–18].

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The aim of the present study was to screen 29 wild edible mushroom species, traditionally consumed by residents in the National Park of North Pindos in Greece for their antioxidant, cytotoxic and pro-apoptotic activity against the bronchial epithelial adenocarcinoma cell line A549 and the identification of chemical compounds which could contribute to the above actions.

Materials and methods

Materials

Piceatannol was purchased from Santa Cruz (California, USA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin–Ciocalteu reagent (FCR), ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin V-FITC/PI (propidium iodide) apoptosis detection kit was purchased from and BD Biosciences Pharmingen (San Diego USA), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Sigma, Chemical Co (MO, USA), while other chemicals of analytical grade were from Merck (Darmstadt, Germany). Solvents (HPLC and LC–MS grade) were obtained from Fisher Scientific.

Mushrooms collection

Twenty-nine different species of wild edible mushrooms, belonging to 14 families, were collected from the National Park Northern Pindos, (North-Western Greece), during the period 2008–2010 (Table 1).

Mushroom species were characterized at the Department of Crop Science of the Agricultural University of Athens. The wild mushrooms species, listed in (Table 1), are indigenous in the area of the National Park of North Pindos. They grow at different vegetation areas starting from altitude 450 m above the sea level and extending up to the highest peak of Smolicas Mountain (2.637 m). The National Park of North Pindos is a woodland area in North-Western Greece, where autochthonous flora has been preserved over the years due to low human activities. This area has a low percentage of population, keeping many of its traditional habits, including the use of wild mushroom species for culinary purposes. The National Park in Northern Pindos district is rich in wild edible mushrooms due to climatic conditions and flora diversity. It consists of various biotopes, such as highland forests or grasslands, which favor the growth of different mushrooms species.

Preparation of mushroom extracts

The mushrooms were thoroughly washed with de-ionized water after removing the inedible parts. Then, the edible parts were mashed, weighted, frozen at − 80 °C for 1 day and lyophilized for 48 h at − 50 °C (LP3 Jouan, France). The dry residue was ground to fine powder using a Kenwood Multi-Mill (Kenwood Ltd, UK) and stored at 4 °C.

For the extraction, 0.4 g of dry powder was suspended into 10 mL of 80% aqueous methanol. The mixture was sonicated at 4 °C, 40 kHz for 1 min, it was allowed for 24 h in the dark at 4 °C, and then it was sonicated again. Afterwards, the samples were centrifuged (Heraeus Labofuge 400, Thermo Scientific, Germany) for 10 min at 800 × g and the supernatants were collected. The remaining sediments were re-extracted twice with 10 mL of 80% aqueous methanol. The combined supernatants were evaporated to dryness and were finally resuspended into 10 mL of 80% aqueous methanol [19]. For cell treatment, 1 mL from each methanol extract was evaporated to dryness prior to the assays, and then was resuspended into 1 mL Ham's F-12K (without FBS).

Table 1 Wild edible mushrooms species included in the present study

Scientific name	Family
Agaricus arvensis Schaeff	Agaricaceae
Agaricus campestris L	Agaricaceae
Agaricus urinascens (F. H. Möller& Jul. Schäff.) Singer	Agaricaceae
Albatrellus pes-caprae (Pers.:Fr.) Pouzar	Albatrellaceae
Amanita caesarea (Scop.) Pers	Amanitaceae
Amanita citrina (Schaeff.) Pers	Amanitaceae
Amanita rubescens (Pers. ex Fr.) Gray	Amanitaceae
Boletus aereus Bull	Boletaceae
Boletus edulis Bull	Boletaceae
Cantharellus cibarius Fr	Cantharellaceae
Cantharellus cinereus (Pers.) Fr	Cantharellaceae
Craterellus cornucopioides (L.) Pers	Cantharellaceae
Hydnum repandum L	Hydnaceae
Hygrophorus marzuolus (Fr.) Bres	Hygrophorceaea
Hygrophorus russula (Schaeff.) Kauffman	Hygrophorceaea
Macrolepiota mastoidea (Fr.) Singer	Lepiotaceae
Macrolepiota procera (Scop.) Singer	Lepiotaceae
Morchella elata Fr	Morchellaceae
Morchella esculenta Fr	Morchellaceae
Morchella conica Pers	Morchellaceae
Flammulina velutipes (Curtis) Singer	Physalacriaceae
Pleurotus eryngii (DC.) Quél	Pleurotaceae
Pleurotus ostreatus (Jacq. ex Fr.) P.Kumm	Pleurotaceae
Ganoderma lucidum (Curtis) P. Karst	Polyporaceae
Lactarius volemus (Fr.) Kuntze	Russulaceae
Russula cyanoxantha (Schaeff.) Fr	Russulaceae
Russula virescens (Schaeff.) Fr	Russulaceae
Calocybe gambosa (Fr.) Donk	Tricholomaceae
Infundibulicybe geotropa (Bull.) Harmaja	Tricholomaceae

Methanol extracts were tested for their cytotoxic and pro-apoptotic activities towards human bronchial epithelial adenocarcinoma cell line (A549 cells), by the MTT assay [20] and flow cytometry, using the Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Biosciences Pharmingen San Diego) [21], respectively.

Antioxidant activity

The antioxidant effect of mushrooms methanol extracts were measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method, according to the procedure described by Sanchez-Moreno et al. [22]. Briefly, 780 µL of DPPH radical (6×10^{-5}molL^{-1}) were added to 20 µL of methanol extract of different mushrooms species at various concentrations (from 1.0, up to10.0 mg mL^{-1}). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm (Shimadzu UV-1601 spectrophotometer, Japan). The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \left[\left(A_c - A_s\right) / A_c\right] \times 100,$$

where A_c was the absorbance of ascorbic acid (control sample) and A_s was the absorbance in the presence of the sample of mushroom extract.

The results were expressed as Median Effective Concentrations, i.e., EC_{50} (mg mL^{-1}) values, which represent the concentration of the mushroom extract, that is required to obtain 50% antioxidant activity. A lower EC_{50} value corresponds to higher antioxidant activity of the mushroom extract. Each sample was analyzed in three replicates.

Determination of total phenolic composition

Total soluble phenols in the extracts were determined with Folin–Ciocalteu reagent according to the method of Slinkard and Singleton, [23], using gallic acid as a standard compound. Briefly, 100 µL of methanol extract was diluted with 4.5 mL distilled water. Then, 100 µL of Folin–Ciocalteau reagent were added and the tube was thoroughly mixed by vortex. After 3 min, 300 µL of Na_2CO_3 (20% w/v) were added and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm. The total phenolic content was expressed as Gallic Acid Equivalents (GAE) in mg per g dry material, using the following equation that was obtained from standard gallic acid graph.

$$\text{Absorbance} = 0.0738 \times \text{gallic acid (mg)} - 0.0368 \left(R^2 : 0.9960\right)$$

Total flavonoids assay

The flavonoid content in the mushroom extract was determined by a colorimetric method described by Zhishen et al. [24, 25]. Briefly, 500 µL methanol extract was mixed with 2 mL distilled water and 150 µL of a 5% w/v $NaNO_2$ solution. After 5 min, 150 µL of a 10% w/v $AlCl_3.H_2O$ solution was added. After 6 min, 1 mL of 1 M NaOH and 1.2 mL distilled water were added to the mixture. The intensity of pink color was measured at 510 nm. The total flavonoids content was expressed as mg quercetin per g dry material, using the following equation that was obtained from standard Quercetin graph.

$$\text{Absorbance} = 0.0032 \times \text{quercetin (mg)} \left(R^2 : 0.9987\right)$$

Cell culture

The human lung adenocarcinoma cell line A549 cells (ATCC CCL-185) were cultured in Ham's F-12K nutrient mixture (Gibco, Paisley, Scotland, United Kingdom) with 10% FBS, 2 mM l-glutamine, 14.2 mM, Na_2CO_3 and antibiotics (10,000 U mL^{-1} penicillin G Sodium, 10,000 µg mL^{-1} streptomycin sulfate, 25 µg mL^{-1}amphotericin B as fungizone) in a humidified incubator (Biotech Galaxy RS, New Brunswick Scientific, USA) with 5% CO_2 at 37 °C. Cell count and viability were determined using Trypan blue exclusion dye. All experiments were performed at the exponential phase of cell growth.

MTT assay for cell viability

Cell survival was tested by the MTT colorimetric assay [20], which is based on measuring the absorbance of formazan produced through the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by living cells. The test was performed into 96-well plate. Each well contained 2×10^4 cells, which were allowed to adhere for 24 h in a 5% CO_2-humidified incubator at 37 °C. Then, the cell supernatant was removed and replaced with increasing concentrations of each extract, namely 1, 2, 5, 10, 15 and 20 mg of dry weight in 200 µL^{-1} Ham's F-12K. After overnight treatment, 20 µL MTT (5 mg mL^{-1} in PBS) were added to each well and allowed for 4 h to react. Then, the culture supernatant was discarded and 100 µL dimethyl sulfoxide (DMSO) were added to each well to terminate the reaction. The plates were shaken for 10 min to dissolve the formazan crystals and the absorbance was measured at 540 nm after 60 min, using microELISA reader (Lambda E, MWG Biotech, Germany). Negative controls included samples without cells, and samples with cells without mushrooms extracts. The viability rate was calculated as follows:

$$\text{Viability (\%)} = 100\% \times A_s / A_c,$$

where A_s mean experimental absorbance and A_c mean control absorbance (cells without mushroom extracts), taking into account the negative controls measurements.

Determination of apoptosis by flow cytometry

A549 cells (1×10^6) were treated with the mushroom extracts, as previously described. Afterwards, they were washed twice with cold PBS and collected by centrifugation. A portion of 1×10^5 cells were suspended into 1 mL binding buffer, containing 5 mL annexin V (binds to phosphatidylserine moieties) and 5 μL of propidium iodide (PI) (marker of necrosis) and incubated for 15 min at 37 °C in the dark [26]. The number of viable, apoptotic and necrotic cells were quantified by flow cytometry (FacsCalibur-Becton Dickinson Franklin Lakes, NJ, USA) from the surface exposure of phosphatidylserine in apoptotic cells using annexin V-FITC/PI apoptosis detection kit (BD Biosciences, Montreal, Quebec, Canada), according to the manufacturer's instructions. The annexin positive/PI negative cells exhibited early apoptosis, annexin positive/PI positive cells exhibited late apoptosis/necrosis, while annexin negative/PI positive cells were necrotic. Data analysis was performed by the Cell Quest software. At least 1×10^4 cells were examined in each sample.

HPLC analysis of mushrooms methanol extracts

Methanol extracts were initially analyzed by preparative High Performance Liquid Chromatography with a Shimadzu HPLC system equipped with an LC-8A diode array detector and a reversed phase column (Ascentis C18 25 cm × 21.2 mm, 10 μm, Sigma–Aldrich, USA). The absorbance was measured at 220 and 280 nm. The mobile phase consisted of (A) 0.1% acetic acid in H_2O, and (B) 0.1% acetic acid in acetonitrile. The elution gradient was 0 min: 92% A; 0–2 min: 90% A; 2–27 min: 70% A; 27–60 min: 10% A; 60–61 min: 0% A; 61–70 min: 0% A; 70–73 min: 92% A [27]. The flow rate was 18.5 mL/min and the injection volume was 5 mL.

LC–MS/MS analysis

The analysis was performed using an ESI-LTQ-ORBITRAP XL unit (Thermo Scientific, Bremen, Germany) coupled with an Accela600 pump and Accela Autosampler. The Orbitrap Unit was operated in negative mode, with a spay voltage of 2.8 kV, while the sheath gas flow rate and auxiliary gas flow rate were adjusted to 30 and 8 arbitrary units, respectively. The capillary voltage and the tube lens voltage were set to 30 and 120 V, respectively. The scan ranged from *m/z* 150 up to 1500. For fragmentation study, a data-dependent scan was performed. The normalized collision energy of the Collision-Induced Dissociation (CID) was set to 35 eV. Separations were performed on Hypersil Gold—C18 column, 100 × 2.1 mm, 1.9 μm (Thermo Scientific, Bremen, Germany). The mobile phase consisted of (A) 0.1% acetic acid in water and (B) 0.1% acetic acid in acetonitrile. The gradient was 0 min: 30%A; 0–10 min: 10%A; 10–12 min: 0%A; 12–20 min: 0%A, 20–23 min: 30%A. The flow rate was 300 μL/min and the injection volume was 10 μL [28].

Data processing for high-resolution MS and MS^2 were carried out by comparing mass spectra of mushroom methanol extracts to that of the standard compounds using the Xcalibur software 2.1.0 by Thermo Scientific.

Statistical analysis

The values are expressed as Means ± SD, while comparison between groups was done by student *t* test and one way ANOVA using the SPSS version 18.0 package. The statistical significance was defined at $P < 0.05$. Each experiment was repeated for at least 3 times using double technical replicas for each condition, unless otherwise stated.

Results and discussion

DPPH free radical scavenging activity

The results concerning the DPPH radical scavenging of mushroom methanol extracts are presented in (Table 2). Results are expressed as EC_{50} (concentrations of extracts in dry weight (mg) per mL methanol that causes 50% of antioxidant activity). As shown in (Table 2), varying degrees of scavenging capacities in mushroom species were observed. The highest antioxidant activities were observed for the methanol extracts of *Ganoderma lucidum, Amanita citrina, Agaricus urinascens* and *Agaricus arvensis* exhibiting the lowest EC_{50} values, namely: 4.0 ± 0.6, 4.0 ± 0.0, 4.9 ± 0.1 and 6.9 ± 0.0 mg dry weight mL^{-1}, respectively, while ascorbic acid showed the highest antioxidant activity (EC_{50}: 0.1 ± 0.1 mg mL^{-1}). In 17 out of a total 29 species of mushrooms tested, the EC_{50} value was greater than 40 mg dry weight mL^{-1}, corresponding to low antioxidant activity. The above data are in consistency with previously published studies. In particular, *Macrolepiota procera, Macrolepiota mastoidea, Agaricus arvensis* and *Cantharellus cibarius* [3, 29, 30], *Morchella esculenta* [7], *Calocybe gambosa* [31], exhibited similar antioxidant activity with that recorded in the present study. According to our best knowledge, no published research studies were concerned with *Agaricus urinascens, Amanita citrina, Hygrophorus marzuolus, Hygrophorus russula*, and *Morchella elata*.

Table 2 %Moisture content, antioxidant activity, total phenolic content, total flavonoid content, cytotoxicity and pro-apoptotic activity of selected wild mushrooms methanol extracts against human cancer cell line A549

Scientific name	%Dry weight	DPPH EC_{50}[a]	Total phenolic content[b]	Total flavonoids[c]	IC_{50} values (mg dry weight /mL)[d]	Annexin V-FITC/PI for cell viability (% ± SD)
Agaricaceae						
Agaricus arvensis	92.4	6.9 ± 0.1	9.2 ± 0.3	1.4 ± 0.1	9.8	24.4 ± 1.3
Agaricus campestris	92.4	13.8 ± 0.3	7.2 ± 0.1	1.7 ± 0.0	15.2	33.2 ± 2.4
Agaricus urinascens	89.0	4.9 ± 0.1	11.1 ± 0.2	1.5 ± 0.1	10.7	-
Albatrellaceae						
Albatrellus pes-caprae	83.6	> 40	2.8 ± 0.2	1.1 ± 0.1	> 20	55.7 ± 0.7
Amanitaceae						
Amanita caesarea	91,8	15.5 ± 1.4	10.1 ± 0.3	0.9 ± 0.0	3.4	19.5 ± 0.4
Amanita citrina	83,4	4.0 ± 0.0	18.5 ± 0.3	7.3 ± 0.3	> 20	57.4 ± 5.3
Amanita rubescens	86.0	19.6 ± 0.4	6.0 ± 0.4	1.9 ± 0.1	14.2	12.8 ± 1.0
Boletaceae						
Boletus aereus	88.5	9.9 ± 0.2	6.0 ± 0.1	1.3 ± 0.1	> 20	40.9 ± 8.8
Boletus edulis	88.9	11.2 ± 0.1	5.7 ± 0.2	2.4 ± 0.2	14.3	23.6 ± 0.6
Cantharellaceae						
Cantharellus cibarius	92.6	> 40	2.0 ± 0.1	0.9 ± 0.1	< 1	8.8 ± 1.9
Cantharellus cinereus	92.7	> 40	1.7 ± 0.1	0.8 ± 0.2	4.4	57.4 ± 0.4
Craterellus cornucopioides	91.3	> 40	1.1 ± 0.1	1.7 ± 0.1	< 1	23.3 ± 0.8
Hydnaceae						
Hydnum repandum	81.2	> 40	1.2 ± 0.1	2.1 ± 0.2	1.0	3.8 ± 6.2
Hygrophoraceae						
Hygrophorus marzuolus	94.0	> 40	1.0 ± 0.1	1.1 ± 0.0	> 20	54.6 ± 3.5
Hygrophorus russula	94.8	> 40	0.7 ± 0.1	1.5 ± 0.1	15.5	6.0 ± 3.3
Lepiotaceae						
Macrolepiota mastoidea	93.4	> 40	3.2 ± 0.2	2.4 ± 0.2	7.8	16.8 ± 6.1
Macrolepiota procera	85.6	20.4 ± 0.4	4.0 ± 0.2	1.0 ± 0.0	16.6	3.4 ± 0.6
Morchellaceae						
Morchella elata	87.8	> 40	5.0 ± 0.2	1.0 ± 0.1	> 20	54.1 ± 0.5
Morchella esculenta	92.5	> 40	4.3 ± 0.1	1.0 ± 0.1	> 20	55.2 ± 0.3
Morchella conica	91.3	20.5 ± 0.8	6.5 ± 0.2	1.4 ± 0.1	> 20	60.1 ± 4.6
Phycalariaceae						
Flammulina velutipes	77.7	> 40	1.3 ± 0.0	0.9 ± 0.1	> 20	79.9 ± 0.5
Pleurotaceae						
Pleurotus eryngii	82.6	> 40	3.3 ± 0.1	0.5 ± 0.1	6.8	18.1 ± 2.3
Pleurotus ostreatus	81.0	> 40	2.4 ± 0.2	0.6 ± 0.1	> 20	57.7 ± 15.0
Polyporaceae						
Ganoderma lucidum	85.0	4.0 ± 0.6	7.7 ± 0.1	3.2 ± 0.1	> 20	28.9 ± 0.2
Russulaceae						
Lactarius volemus	90.4	> 40	1.9 ± 0.1	1.0 ± 0.1	> 20	58.9 ± 3.8
Russula cyanoxantha	89.7	> 40	2.8 ± 0.2	1.7 ± 0.1	8.2	33.6 ± 5.2
Russula virescens	89.9	> 40	2.2 ± 0.1	0.8 ± 0.1	14.9	32.5 ± 22.9
Tricholomataceae						
Calocybe gambosa	82.2	> 40	4.0 ± 0.2	1.1 ± 0.1	1.6	46.6 ± 1.9
Infundibulicybe geotropa	94.0	36.6 ± 0.9	2.6 ± 0.2	3.3 ± 0.2	9.4	31.2 ± 2.5
Ascorbic acid		0.1 ± 0.1				

[a]Concentrations of extracts in mg dry mL^{-1} that cause 50% of antioxidant activity

[b]mg GAE/g dry weight (SD ± Mean)

[c]mg quercetin/g dry weight (SD ± Mean)

[d]Results are expressed as IC_{50} (concentrations of extracts in mg dry weight mL^{-1} that cause 50% of cytotoxicity)

Determination of total phenolic composition

Total phenolic composition of mushrooms extracts are shown in (Table 2). Among the studied species, *Amanita citrina* showed the highest total phenolic content, followed by *Amanita caesarea* and *Agaricus arvensis*. Other studied species exhibited lower phenolic concentrations. Particularly, *Hygrophorus russula, Hygrophorus marzuolus, Cantharellus cinereus, Craterellus cornucopioides, Hydnum repandum*, and *Flammulina velutipes*.

The above results are in consistency with previously published studies. In particular, similar total phenolic contents with those measured in the present study were reported for *Macrolepiota procera, Macrolepiota mastoidea, Agaricus arvensis, Calocybe gambosa, Boletus edulis, Craterellus cornucopioides, Cantharellus cibarius* [3, 29, 30], *Pleurotus ostreatus* [32], *Boletus edulis* [33, 34] and *Hydnum repandum* [35, 36]. To our best knowledge, no published studies addressed *Agaricus urinascens, Amanita citrina, Hygrophorus marzuolus, Hygrophorus russula* and *Morchella elata*.

Total flavonoid content

Only a few data have been reported with respect to the flavonoids content of wild edible mushrooms. As shown in (Table 2), the concentration in flavonoids varies depending on the mushrooms species. The majority of the studied species were characterized by low flavonoid concentration; however, *Amanita citrina* apart from the highest total phenolic content also exhibited the highest flavonoid levels.

Our results are in consistency with previously published studies on the following species: *Morchella esculenta, Morchella elata* and *Morchella conica, Boletus aereus, Cantharellus cibarius, Ganoderma lucidum, Agaricus campestris* [37–39], using quercetin as standard compound and for *Macrolepiota procera, Macrolepiota mastoidea, Calocybe gambosa, Cantharellus cibarius, Craterellus cornucopioides, Boletus edulis, Agaricus arvensis, Hygrophorus marzuolus* and *Pleurotus ostreatus* [3, 8, 40], using catechin as a standard compound. According to our best knowledge, the total flavonoids content of *Agaricus urinascens, Amanita citrina, Hygrophorus russula* and *Morchella elata* was not addressed in other published studies.

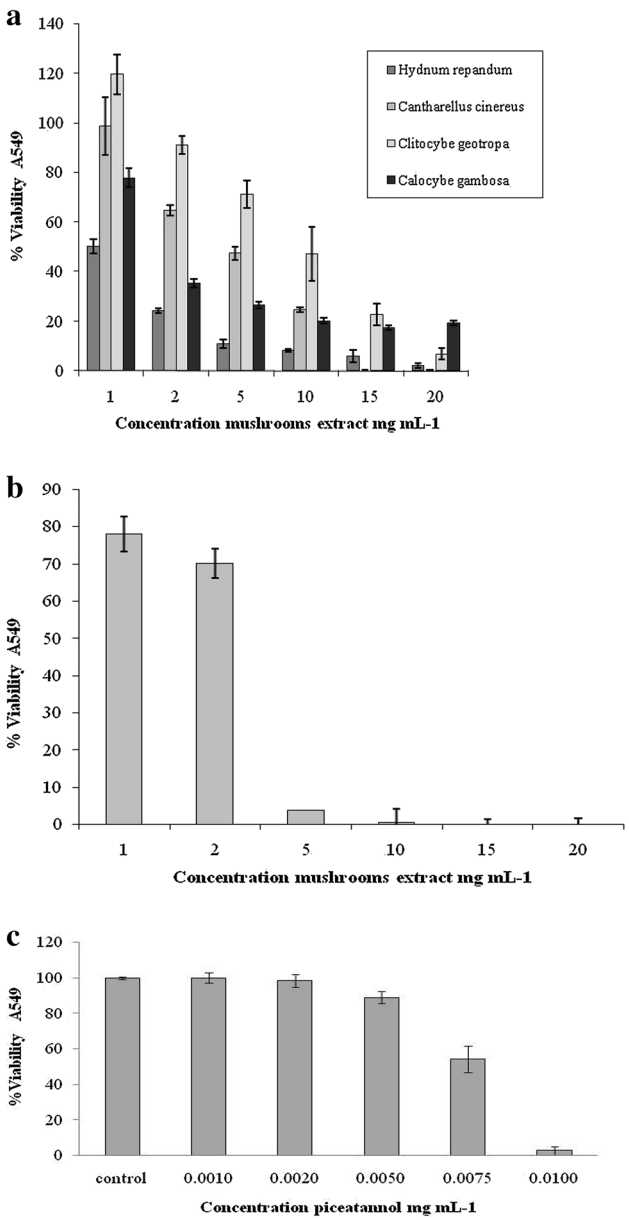


Fig. 1 Viability (%) of A549 cells after treatment with increasing concentrations of methanol extracts obtained from various wild edible mushrooms, using the MTT assay (**a**), *Cantharellus cibarius* (**b**) and authentic piceatannol (**c**). Each experiment was repeated three times ($n=3$)

In vitro cytotoxic effect of methanolic extracts on A549 human cells

Mushroom extracts were tested for their cytotoxic activity against human lung adenocarcinoma cell line A549 (ATCC CCL-185). Cell viability was significantly affected after treatment with most of the mushroom extracts tested, showing dose-dependence (Fig. 1a). The most prominent effect was observed for *Cantharellus cibarius* extracts, which, at concentrations of 1, 2, 5, 10, 15 and 20 mg dry weight mushroom per mL medium, led to a percentage of cell death of 0.00, 0.03, 0.37, 3.63, 70.15 and 78.02%, respectively (Fig. 1b). The cytotoxic activities of the mushroom extracts of this study, expressed as IC_{50} values, are presented in (Table 2). Wild mushrooms with the highest cytotoxicity, and consequently with the lowest IC_{50} value against the A549 cell line, were *Cantharellus cibarius* and *Craterellus cornucopioides* ($IC_{50} < 1$ mg mL^{-1}), *Hydnum repandum*

(1.0 mg mL^{-1}), *Amanita caesarea* (IC_{50} of 3.4 mg mL^{-1}) and *Cantharellus cinereus* (IC_{50} of 4.6 mg mL^{-1}). The cytoxicity of authentic piceatannol was also tested against cell line A549. A549 cells viability was significantly reduced after treatment with extremely low concentrations of authentic piceatannol (Fig. 1c).

Pro-apoptotic activity of wild mushroom extracts on A549 cells

Extracts from 29 different species of wild edible mushrooms, belonging to 14 families, were evaluated for their pro-apoptotic effect on the cancer cell line A549 by flow cytometry (Table 2). As shown in (Fig. 2a) and (Fig. 2b, c), *Cantharellus cibarius* caused 91.23% cell death (apoptosis and necrosis) *Amanita caesarea* 80.52%, *Amanita rubescens* 87.24%, *Hydnum repandum* 96.22%, *Pleurotus eryngii* 81.91%, *Hygrophorus russula* 94.01% *Macrolepiota procera* 96.61% and *Macrolepiota mastoidea* 83.20%. These levels of cytotoxicity (over 80%) against A549 cell line are considered impressively high.

Impressively high cytotoxicity and apoptosis–necrosis against the human epithelial adenocarcinoma cell line (A549 cells), were imposed by methanol extracts from six mushroom species, particularly *Cantharellus cibarius* (cytotoxicity 100.08% and cell death 91.23%, respectively), *Craterellus cornucopioides* (98.06 and 76.73%) *Hydnum repandum* (97.92 and 96.22%), *Cantharellus cinereus* (100.44 and 42.58%), *Macrolepiota procera* (88.24 and 96.61%) and *Macrolepiota mastoidea* (87.45 and 83.20%). The species *Cantharellus cibarius, Cantharellus. cinereus, Craterellus cornucopioides* and *Hydnum repandum*, which belong to the order Cantharellales, exhibited similar levels of cell death efficiency. To identify whether a compound common to the above mushrooms was the active ingredient, we performed fractionation of the methanol extracts.

HPLC analysis

In species with high cytotoxic activity (> 80%) belonging to the order of Cantharellales such as: *Cantharellus cibarius, Cantharellus cinereus, Craterellus cornucopioides* and *Hydnum repandum*, fractionation of the methanol extracts was performed by preparative HPLC analysis, to identify the active compounds with cytotoxic effects on A549 human cell line.

The chromatographic analysis of the above four species of mushrooms revealed that only the fractions collected by preparative HPLC (Fig. 3a) at retention times between 61.00 and 65.00 min (at a wavelength of 220 nm) exhibited potent in vitro cytotoxic activity against A549 cell line, using the MTT assay (Fig. 3b). For this purpose, these fractions were gathered for further analysis.

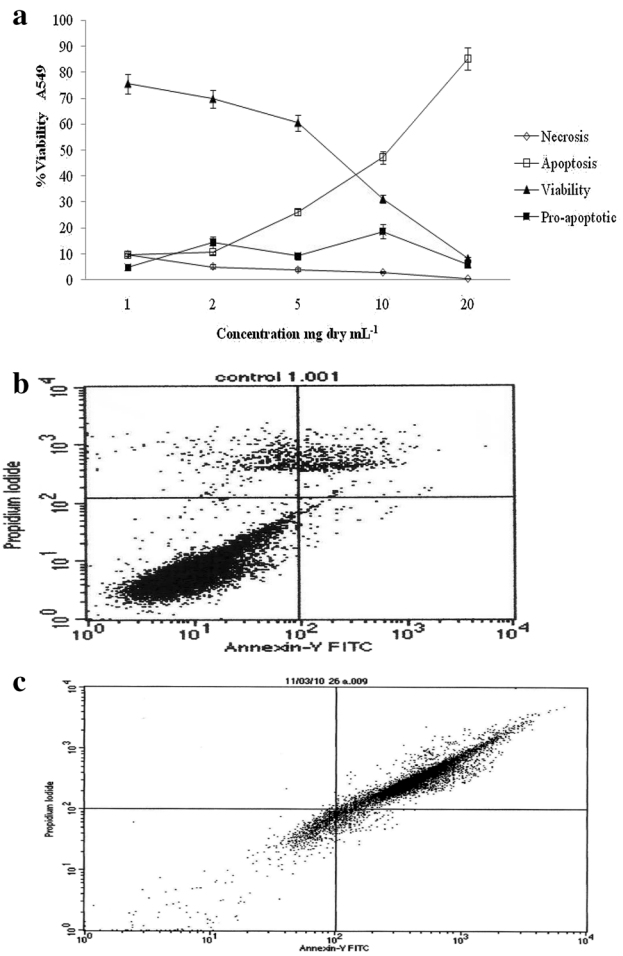


Fig. 2 **a** Pro-apoptotic activity of *Cantharellus cibarius* methanol extracts on A549 cancer cell line using flow cytometry. Cells were treated with 1, 2, 5, 10 and 20 mg dry weight mL^{-1} for 24 h and stained with annexin V/PI. Results of early apoptotic, late apoptotic/necrotic, necrotic cells and cell death are expressed as percentage of total cells, measured for 10,000 events. Representative pattern of flow cytometric analysis: **b** control-untreated A549 cells and **c** A549 cells treated with 20 mg/mL methanol extract of *Cantharellus cibarius*, for 24 h. Each experiment was repeated three times ($n = 3$)

Analysis of preparative HPLC fractions with LC–MS/MS

The preparative HPLC fractions were further analyzed by LC–MS/MS, to identify the possible chemical compounds derived from the mushrooms extracts which possessed a cytotoxic effect on A549 cell line.

The careful study of the mass spectra for the fractions which exhibited potent in vitro cytotoxicity against A549 cell line revealed that the molecular ion $[M–H]^-$ at m/z 243.0656 was detected in all these fractions. This specific molecular ion was common for the entire mushroom extracts of the order Cantharellales, exhibiting high cytotoxicity.

Furthermore, the molecular ion $[M–H]^-$ at *m/z* 243.0656 was also not detected in mushroom species fractions which exhibited no cytotoxic activity against A549 cell line.

The identification of this compound was confirmed using LC/MS and MS^2 analysis by comparison with data available in the literature and those of authentic standards. The molecular ion was identified as piceatannol which belongs to natural stilbenes (Fig. 4a, b).

Conclusion

In conclusion, although a few studies addressed the antiproliferative activity of mushrooms [41, 42], this is the first report on methanol extracts from wild edible mushrooms from Greece and particularly from the National park of North Pindos in North-Western Greece. The present study represents a screen for antioxidant activity, cytotoxicity and antiproliferative activity of methanol extracts of 29 different wild edible mushrooms species that are consumed traditionally by residents in Greece.

According to our results, the order Cantharellales possesses strong antiproliferative activity. To our best knowledge piceatannol, an analog of resveratrol, is identified for the first time in methanol extracts of wild edible mushrooms. Few studies have been addressed the biological actions of piceatannol [43], such as ROS scavenging [44] or a potent induction of apoptosis in human cells [43, 45]. We suggest that piceatannol exhibits a potent antiproliferative action in cojunction with other compounds, the nature of which need to be investigated in future experiments. The results of the

Fig. 4 Representative MS^2 spectra of the parent molecular ion ▶ $[M–H]^-$ at *m/z* 243.0656 for (**a**) standard piceatannol solution and **b** the fraction of *Cantharellus cibarius* which exhibited cytoxicity against A549 cells. Each experiment was repeated three times ($n = 3$)

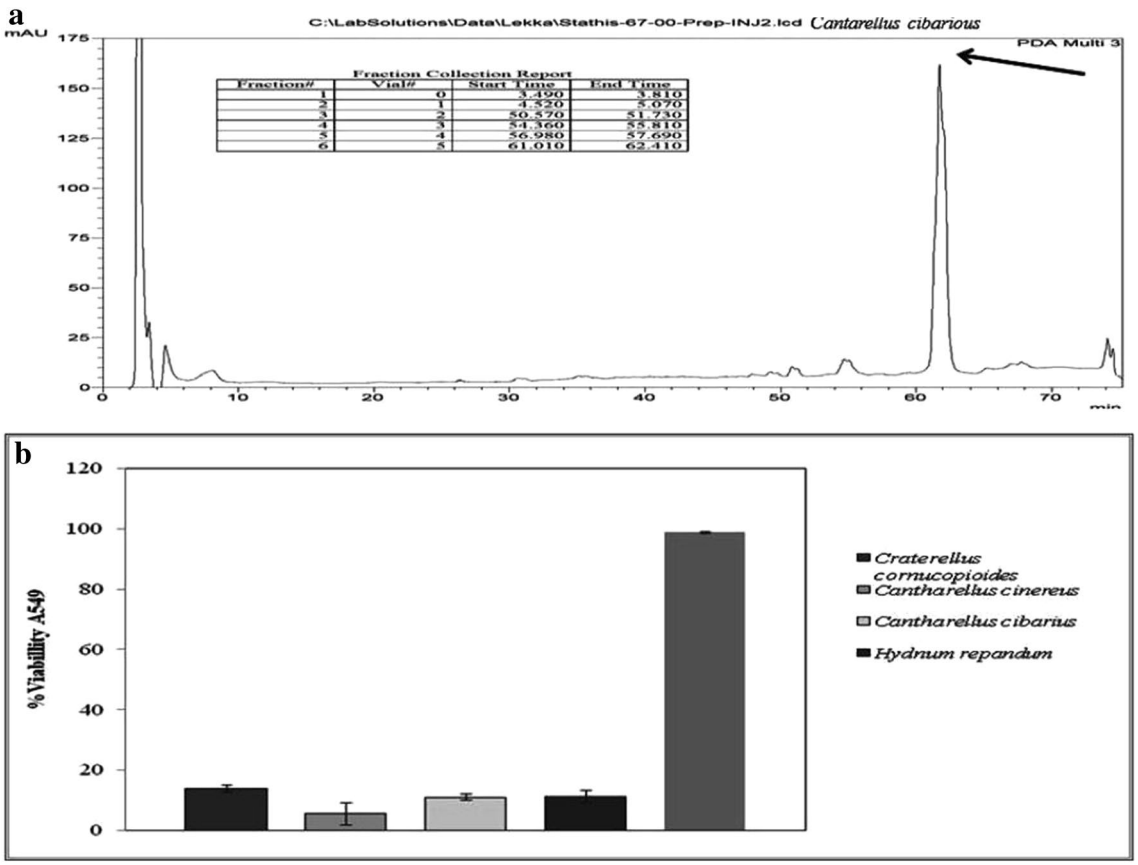
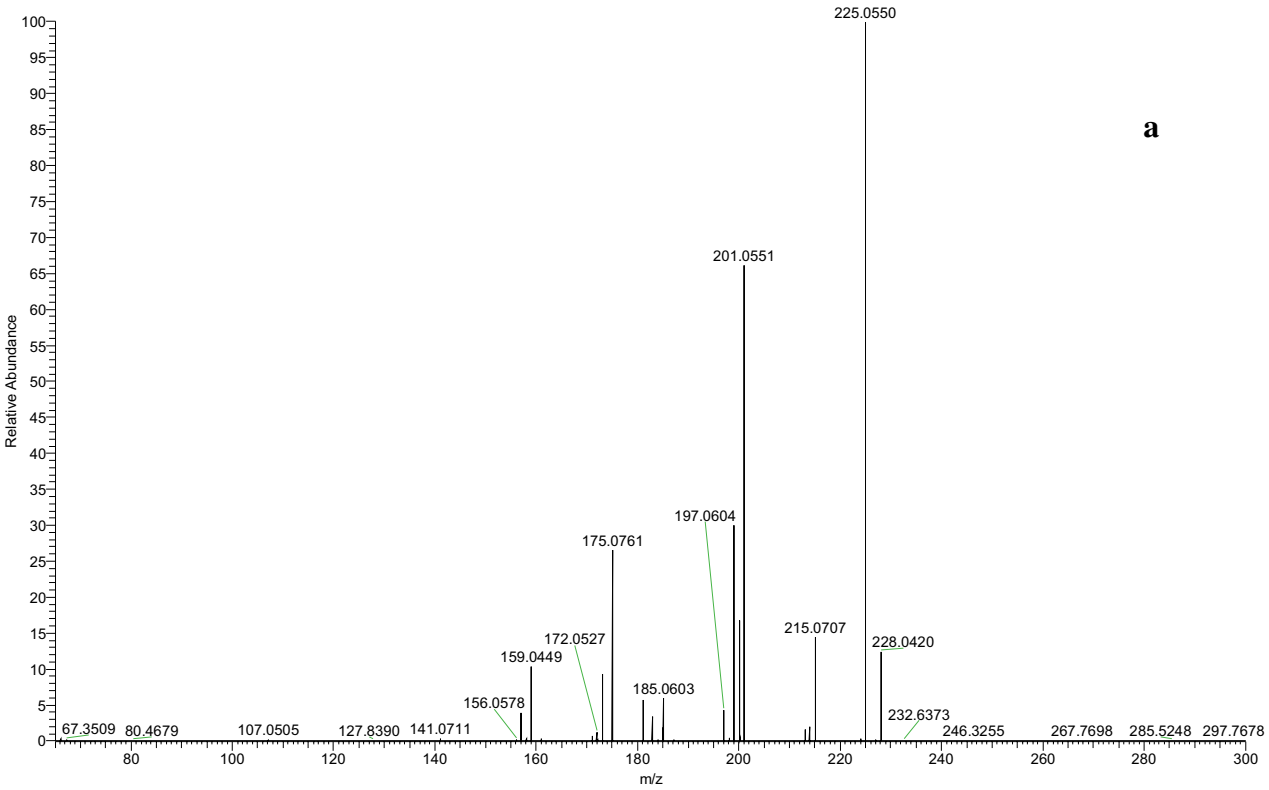
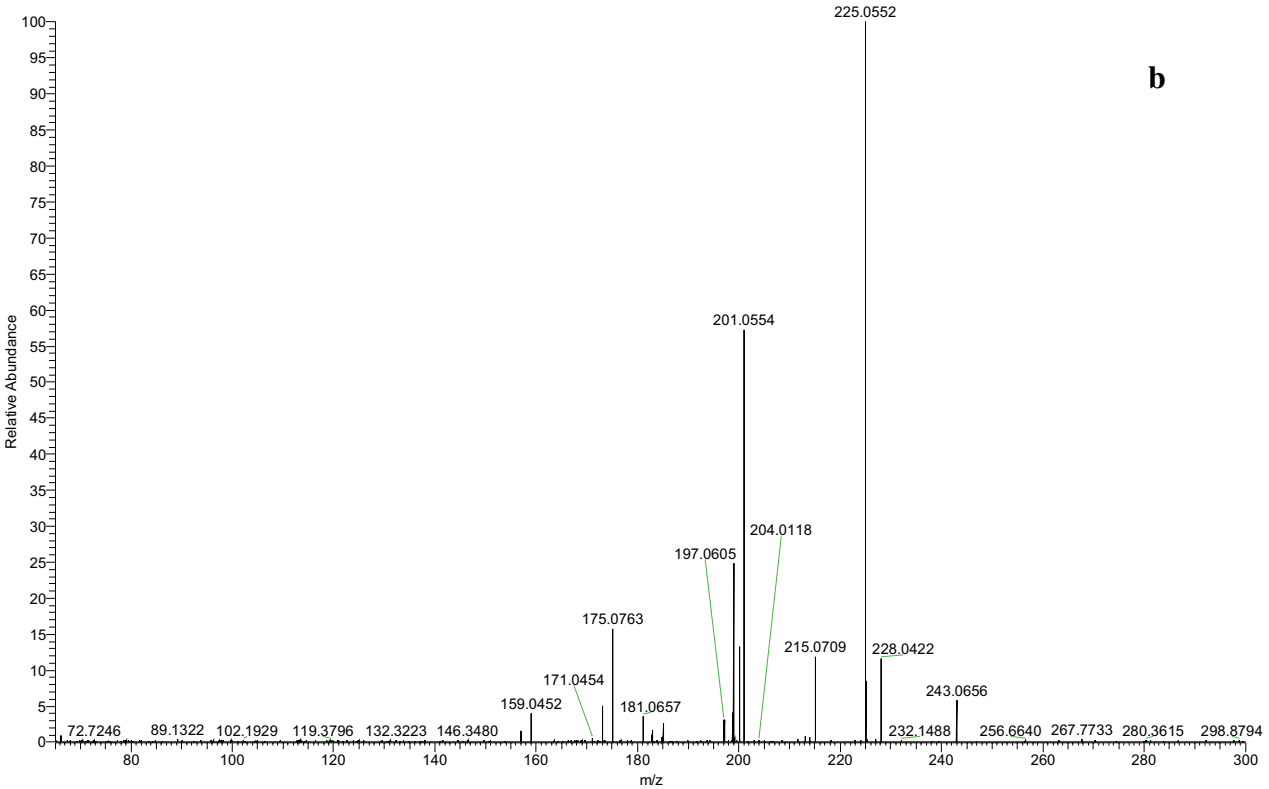


Fig. 3 **a** Representative preparative HPLC chromatographic pattern of the methanol extract of *Cantharellus cibarius*. The absorbance of each sample solution was measured at 220 nm. The mobile phase consisted of (**a**) water + 0.1% acetic acid and **b** acetonitrile + 0.1% acetic acid. The gradient was 0 min, 92% A; 0–2 min, 90% A; 2–27 min, 70% A; 27–60 min, 10% A, 60–61 min, 0% A; 61–70 min, 0% A; 70–73 min, 92% A. The flow rate was 18.5 mL/min and the injection volume was 5 mL. **b** In vitro cytotoxic effect of the preparative HPLC fractions of the methanol extracts of *Cantharellales*. The fractions were collected at retention times between 61.00 and 65.00 min and the cytotoxic effect was expressed as % viability of A549 cells. Each experiment was repeated three times ($n = 3$)


a
Relative Abundance
m/z
225.0550
201.0551
197.0604
175.0761
215.0707
228.0420
172.0527
159.0449
185.0603
156.0578
232.6373
67.3509
80.4679
107.0505
127.8390
141.0711
246.3255
267.7698
285.5248
297.7678


b
Relative Abundance
m/z
225.0552
201.0554
204.0118
197.0605
175.0763
215.0709
228.0422
171.0454
243.0656
159.0452
181.0657
72.7246
89.1322
102.1929
119.3796
132.3223
146.3480
232.1488
256.6640
267.7733
280.3615
298.8794

present study indicate that the tested mushrooms are promising sources of bioactive compounds. Future work should also aim to clarify the mechanisms of action of these mushrooms extracts on human tumor cell lines.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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