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Antibacterial and Antitumor Activity of Crude Methanolic Extracts from Various Macrofungi Species

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Abstract

Mushrooms are considered to be an important natural resource for the investigation of new compounds with antimicrobial, anti-tumor or immunomodulatory effects because of their secondary metabolites and degrading enzymes. In this study, antibacterial and antitumor potential of *Cantharellus cibarius, Clitocybe geotropa, Gyromitra esculenta, Lactarius delicious, Melanoleuca exscissa, Ramaria flava, Sarcosphaera crassa, Morchella sp., Stereum hirsutum* and *Trametes versicolor* mushrooms collected from Mugla region have been investigated with disc diffusion method and MTT assay, respectively. Among all mushroom extracts, *M. exscissa* extract showed the highest antimicrobial effect on *E. coli* (22 mm, 80 µg/disc inhibition zone). It was observed that 8 out of 16 mushroom extracts did not show any effect on all test microorganisms, the others formed up to 9 mm inhibition zone in diameters. In addition, a total of 16 mushroom extracts (400 µg/ml) were tested on 5 different cancer cell lines. It was detected that some of the extracts decreased cell proliferation but the others increased. The extracts from 7 different species of *Morchella* showed cytotoxic effect on the K-562 cell line. However, all of the extracts from other mushrooms exhibited cytotoxic effects on A-549 cell line. The highest cytotoxic effect on cancer cells was observed with *M. deliciosa* extract on CCC-221 cell line (79%) and with *M. distants* extract on K-562 cell line (72%). It was found that *Trametes versicolor* extract was the most effective extract among other mushroom species and it caused 72% cytotoxicity on A-549 cell line. Consequently, these mushrooms may be a potential source in the development of antitumor compounds. Active substances and mechanism of antitumor activity will be clarified in a future study.

Keywords: Antibacterial activity, Antitumor activity, Morchella, Macrofungi

INTRODUCTION

Mushrooms, known as their edible, medicinal and poisonous properties, have been a valuable nutrient and used as a medicinal source since ancient times [1]. They have played an important role in traditional eastern medicine in Japan, China, Korea and other Asian countries [2]. The medicinal property of macrofungi is based on bioactive molecules such as polysaccharides, glycoproteins, proteoglycans, terpenoids, fatty acids, proteins and lectins. Many anti-tumor and immunomodulator agents have been derived from these components [3].

In the treatment of bacterial diseases, drug resistance is a serious problem caused by incorrect application of antimicrobial agents. At this point, new antibiotics derived from different fungi extracts attribute a potential source to deal with drug resistant bacteria strains. Even though antibiotics obtained from microfungi, such as penicilin, have been widely available on the market, there have been numerous investigations about antibiotics from macrofungi. For instance, ganomycins produced by *Ganoderma* macrofungus showed antimicrobial activity against multi drug resistant bacteria *Staphylococcus aureus* [4]. For this reason, macrofungi should be searched in the production of new antibiotics.

Cancer is one of the common and malignant disease worldwide. Numerous research have being carried out to explore natural products and effective biological agents for the treatment of cancer [5]. Mushrooms have been a good candidate to prevent the development of cancer. In the literature, there have been many studies about isolation, structural characteristics and antitumor activity of mushrooms [6, 7]. The antitumor studies have been focused on *Basidiomycetes* mushrooms due to their biologically active components, especially polysaccharides, possesed in fruit bodies, mycelium or culture broth. There have been found 651 species of *Basidiomycetes* with antitumor activity [2].

The macrofungi have distinctive fruiting bodies and belong to classes of *Ascomycetes* or *Basidiomycetes* [8]. All the mushroom species except *Morchella* used in this research belong to class of *Basidiomycetes*. Up to now, estimated number of macrofungi is around 140,000. Of these, 2000 species are edible and around 200 have been used for food and medicinal purposes [9]. Macrofungi have already been proven to contain so many bioactive metabolites [10]. Even though main chemical composition of many mushroom species is known in the literature, the level and quality of phytochemicals in mushroom are significantly affected by environmental conditions such as UV radiation, air pollution, parasites, and temperature. Therefore, we aimed to investigate antibacterial and antitumor effects of some edible and parasite mushrooms collected from Mugla region of Turkey in this study.

MATERIALS AND METHODS

Mushroom Material

The edible and parasitic fungi used in this study (*Ascomycetes* or *Basidiomycetes*) were collected from Mugla provience (Turkey). Mushroom species were identified by Dr. Hayrunisa Baş Sermenli in Cryptogam Laboratory of MSK university.

Preparation of Mushroom Extracts

The fresh edible mushrooms (*Cantharellus cibarius, Clitocybe geotropa, Gyromitra esculenta, Lactarius delicious, Melanoleuca exscissa, Ramaria flava, Sarcosphaera crassa, Morchella sp(1), Morchella angusticeps, Morchella rotunda, Morhcella eximia, Morchella deliciosa, Morchella distans, Morchella sp(2), Morchella esculenta*) and parasitic mushrooms (*Stereum hirsutum, Trametes versicolor)* were dried at 40 °C in the oven overnight. Dry mushroom samples (30 g) were mixed with 300 ml of methanol as a solvent. The samples were extracted for 7 days at 30 °C with 150 rpm shaking conditions. The solvent was removed periodically and methanol was added to the remaining sample [11, 12]. The extracts were filtered using Whatman filter paper no.4. After that, the solvent was removed using a rotary evaporator under vacuum at 45 °C. The extracts were stored at 4 °C until processed.

Bacterial Strains

Two Gram-negative bacteria *Escherichia coli* (ATCC 11230), *Pseudomonas aeruginosa* (ATCC 29212), and four Gram-positive bacteria *Staphylococcus aureus* (ATCC 6538/P), *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (NRRLB-4375) and *Streptomyces albus* (CIP104432) strains were used for antibacterial assay.

Determination of Antibacterial Activity

To determine the antibacterial activity, different concentrations of mushroom extracts were tested by disc diffusion method. The microorganisms were grown in Nutrient Broth for 24 h, at 37 °C. The bacterial cells were diluted with sterile saline to adjust the turbidity of suspension to 0.5 Mc Farland standart. The culture was poured into Muller Hinton agar. Then, 20 µl of each extract (4 mg/ml) were absorbed into the 6 mm sterile discs (Oxoid) and placed onto the dishes. Among commercial antibiotics; vancomycin (30 µg/ml) gentamycin (10 µg/disc) and meropenem were used as positive controls whereas methanol was used as negative control. After incubation for 2 hours at 4 °C, the bacteria were then incubated for 24 h at 37 °C. After that, the diameter of the inhibition zones including the disc were measured in mm.

Cell Lines and Culture Conditions

K-562 (chronic myeloid leukemia), DU-145 and PC-3 (prostate carcinoma), A-549 (lung carcinoma), CCC-221 (colon carcinoma), MCF-7 (breast carcinoma) and BEAS-2B (healty epithelium cell) cell lines were obtained from Dr. Yusuf Baran (Izmir Institue of Technology, Turkey). The cell lines were cultured in Roswell Park Memorial Institute Medium (RPMI-1640) supplemented with heat-inactivated 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml) (Biochrom, Germany). The cells were incubated at 37°C in 5% CO_2 and 95% air in a humidified incubator.

Cytotoxicity Assay by MTT

The antitumor effect of mushroom extracts was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-dipenyltetrazolium bromide] assay (Applichem, USA). This assay detects the reduction of MTT to blue formazan product by mitochondrial dehydrogenase which reflects the function of mitochondria and cell viability [13]. Exponentially growing cells at $2x10^4$ cells/ml were plated in triplicate into 96-well plates (Greiner, Germany) in 200 µl of growth medium and incubated for 24 hr before the addition of extracts. Mushroom extracts (8 mg/ml) was dissolved in 10% DMSO and added to the cell culture at final concentration of 400 µg/ml to be tested aganist six cell lines. Cells were incubated for 72 hr at 37 °C in 5% CO_2 incubator. After that, 10 µl of PBS containing 5 mg/ml MTT was added into each well. After 4 hr incubation, the medium was discarded and formazan blue crystals formed in the cells were dissolved in 100 µl DMSO. Reduced MTT was quantified by reading the absorbance at 540 nm on a microplate reader (Thermo Multiskan Microplate Spectrophotometer). Cytotoxic effects of the tested extracts were determined by comparing the optical density of the treated cells aganist the optical density of the untreated cells.

Statistical Analysis

Data were analysed by using SPSS soft ware package and Microsoft Excel. Multiple comparisons of treatments were performed with one-way ANOVA and Dunnet post-hoc tests. A difference was considered to have significance at $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Data are presented as mean ± SD of three replicates.

RESULTS AND DISCUSSION

Antimicrobial Activity

The crude extracts of 16 macrofungi exhibited different level of antibacterial activities against 6 different bacteria. According to the results of disc diffusion assay, extract from *M. exscissa* showed the highest antibacterial activity against *E. coli* by forming 22 mm inhibition zone (Table 1). However, maximum inhibition zone displayed by other extracts was less than 9 mm. For example, 7 mm and 8 mm inhibition zones against *P. aeruginosa* and *B. subtilis* were observed, respectively with *C. cibarius* extract. The extract of *S. hirsutum* created an inhibition zone 9 mm for *B. subtilis*, 8mm for both *P. aeruginosa* and *M. luteus*. The extract of *T. versicolor* had low antibacterial activity by forming 7 mm of inhibition zone against *P. aeruginosa, S. aureus* and *M. luteus*. Similarly, inhibition zones between 7 and 9 mm was observed in *Morchella* groups of *M. sp (1), M. angusticeps, M. rotunda, M. deliciosa* and *M. distans* against *P. aeruginosa.* Compared to other bacteria used, a Gram negative bacteria *P. aeruginosa* appeared to be more susceptible to inhibitory effects of mushroom extracts (Table 1). In other words, most of the extracts had some degree of antibacterial activity against *P. aeruginosa*. However, most of the extracts did not show any antibacterial activity against six different bacteria used (Table 1).

Based on the literature, medicinal mushrooms contain a number of biologically active compounds and classified as high molecular weight compounds such as polysaccharides, proteins; and low molecular weight substances such as terpenoids, polyketides, alkaloids, and metabolites derived from non ribosomal peptide synthesis. Macrofungi extract

Table 1. Antibacterial activities of various mushroom extracts

Bacteria / Macrofungi	Zone of inhibition (mm)					
	E. coli	*P. aeruginosa*	*S. aureus*	*B. subtilis*	*S. albus*	*M. luteus*
C. cibarius	-	7	-	8	-	-
C. geotropa	-	-	-	-	-	-
G. esculenta	-	-	-	-	-	-
L. delicious	-	-	-	-	-	-
M. exscissa	22	-	-	-	-	-
R. flavescens	-	-	-	-	-	-
S. crassa	-	-	-	-	-	-
S. hirsitum	-	8	-	9	-	8
T. versicolor	-	7	7	-	-	7
Morchella sp.1	-	9	-	-	-	-
M. angusticeps	-	8	-	-	-	-
M. rotunda	-	8	-	-	-	-
M. eximia	-	-	-	-	-	-
M. deliciosa	-	8	-	-	-	-
M. distans	-	9	-	-	-	-
Morchella sp.2	-	-	-	-	-	-
M. esculenta	-	-	-	-	-	-
Antibiotic	23	23	16	22	21	26

(-): No inhibition; Results are means of three replicates.

from different species were tested against different bacteria [10]. Especially extracts from *Ganoderma* species are well documented for antimicrobial potential. For example, ganomycin A and ganomycin B which are hydroquinones isolated from *G. pfeiferi* exhibited antimicrobial activity against various Gram-positive and Gram-negative bacteria [14].

Akyüz et al. [15] examined antimicrobial activity of some edible mushrooms from Eastern and Southern part of Turkey. Even though they used different mushroom species and bacteria than ours, antimicrobial activity of most of the mushroom extracts ranged from 7 mm and 9.5 mm of inhibition zone. Similarly, antimicrobial activity of mushroom extracts from Mugla region caused inhibition zone between 7 mm and 9 mm. In addition, Altuner and Akata [16] examined antimicrobial activity of some macrofungi extracts and found that all of them had antimicrobial activity against *Shigella flexneri* but none of them was effective against *E. coli*. Moreover, in the study of Nedelkoski et al. [17], six wild mushroom species different than ours exhibited more potent inhibitory effect on the growth of bacteria than on fungi and the highest antimicrobial activity was observed with *Phellinus igniarius* mushroom.

Stereum hirsutum is a wood decay mushroom and its mycelia has being used as functional foods. The fermented mycelia of *S. hirsutum* has been reported to contain various bioactive secondary metabolites [18]. In the study of Ma et al. [19], two new benzoate derivatives isolated from the mycelia of *S. hirsutum* exhibited antimicrobial activity against *S. aureus* with the MIC value of 25 µg/ml. However, crude methanolic extract applied at 80 µg/disc did not cause antimicrobial activity against *S. aureus* in this present study, whereas it resulted in 8, 9 and 8 mm inhibition zone against *P. aeruginosa*, *B. subtilis* and *M. luteus*, respectively (Table 1). It appears that fruting body and mycelia of the mushroom contain different compounds and pure fraction of benzoate derivatives obtained after fractination of the ethyl acetate extraction of fermented rice subtrate with mycelia are more effective on antimicrobial activity compared to crude extracts of fruting body.

No antimicrobial activity was detected with the extracts of edible mushroom *R. flava* in this study. Unlike our study, antimicrobial activity of *R. flava* was reported against *E. coli* and *S. aureus;* and *S. aureus* found to be more susceptible than *E. coli* [20]. Difference in these results is most probably due to the solvent used for the assay. Indeed, they used ethanol extract at 5 mg/well concentration whereas we used methanolic extract at 80 µg/disc. Similar to our result, Giri et al. [21] did not find antimicrobial activity of methanolic crude extract of *R. botrytis* at 1 mg/disc against *S. aureus*.

Biological activity of *Lactarius vellereus* was investigated by Doğan and Aydın [22]. They observed the minimum inhibitory effect with acetone extract (19 µg/ml) against *Klebsiella pnemoniae*. In addition, Bala et al. [23] found that ethanol extracts were more effective against *S. aureus* than *E. coli*. When aqueous and methanolic extracts of *T. hirsuta* were examined against *S. aureus*, the maximum antibacterial activity was observed with aqueous extract.

Kozarski et al. [24] examined antimicrobial activity of methanolic extracts of *C. cibarius* and found 9.5 mm and 8.5 mm inhibition zone against *S. aureus* and *E. coli*, respectively. Even though we also used the methanolic extract of *C. cibarius*, no antibacterial activity was observed against these bacteria. The difference could be due to the amount of extract used in the assay in that they used the extract at 1 mg/disc whereas we used 80 µg/disc.

Antimicrobial effect of *Morchella* species was investigated by different research group. For example, Badshah et al. [25] examined antimicrobial effect of methanolic, chloroform and ethanolic extracts of *M. esculenta* and reported that maximum antibacterial activity

with 23.5 mm inhibition zone against *E. coli* was observed at 30 mg/ml concentration of methanolic extract. In addition, Turkuglu et al. [26] reported a narrow antibacterial spectrum against test microorganism with ethanol extract of *M. conica*. Morever, Kalyoncu et al. [27] studied the antimicrobial effect of ethanolic mycelia extracts from 5 different *Morchella* species and found that some *Morchella* species exhibited antimicrobial activity with 8 mm inhibition zone against *E. coli* and *S. aureus* but others did not.

As a result, current research suggest that macrofungi are attractive source for the novel antimicrobial agents and the degree of antimicrobial activity of each mushroom species depends on the type of solvent for extraction, test microorganism, different test methods, amount of extract used in the assay and chemical profiles of the macrofungi. In addition, antibacterial activities of most of the macrofungi extracts examined in the current study are similar or lower than those reported in the literature. However, to our knowledge there is no previous report for the antimicrobial effect of *M. exscissa* against *E. coli*, and this result is presented here for the first time.

Antitumor activity

A compound that is capable of preventing tumor formation is called antitumor agent. Because many recent studies indicate that bioactive metabolites from mushrooms control and prevent the development of tumors, there may be potantially novel compounds still to be discovered. Therefore, this current study examines the cytotoxic effects of macrofungi belonging to different species on various cancer cell lines. We used the extracts at high concentration (400 µg/ml). Control containing the appropriate volumes of blank solutions was included in the assay and cytotoxicity of the extracts were normalized with that of the control.

When cytotoxic effect of 7 different *Morchella* species was investigated, varying degree of cytotoxicity on the cell lines was observed (Figure 1). For example, extracts from some of the *Morchella* species caused cytotoxicity but others resulted in cell proliferation on DU-145, PC-3, CCC-221 and A-549 cell lines. However, extracts from all *Morchella* species displayed only cytotoxic effect ranged between 36-72% on K562 cell line (Figure 1 E). The highest cytotoxicity (72%) on K562 cells was obtained with *M. distance* while the lowest cytotoxicity (36%) with *M. esculenta*. Among all cancer cell lines examined, *M. deliciosa* exerted the highest cytotoxicity (79%) on CCC-221 cell line (Figure 1, C). Although cytotoxicity of *M. deliciosa* extract was respectively 32% and 42% on DU-145 cell and PC-3 lines (Figure 1A,B), it induced the cell proliferation in A-549 cells (Figure 1D). The most cytotoxic effects (56% and 54%) on A-549 cells were observed with the extracts from *M. eximia* and *M. esculenta* (Figure1, D).

In addition to extracts of *Morchella* species, the extracts from 9 different macrofungi species were examined for their cytotoxic potential on A-549 cells (Figure2, A). Extracts from *C. cibarius*, and *T. versicolor* were tested on CCC-221 and PC-3 cells as well (Figure 2, B). *T. versicolor* extract was found to be the most effective extract among other mushroom species and it caused 72% cytotoxicity on A-549 cells. The extract from *C. geotropa* and *R. flava* resulted in 64% and 67% cytotoxicity on the same cell line (Figure 2, A). Moreover, extract of *C. cibarius* caused 62% cytotoxicity on CCC-221 cells whereas 52% cytotoxicity on PC-3 cells (Figure 2, B). Finally, *T. versicolor* extract exhibited 63% cytotoxicity on PC-3 cells but 51% cytotoxicity on CCC-221 cells.

Because many polysaccharides extracted from mushrooms displayed antitumor and immunostimulating activities [7, 10], a number of research has being conducted to explore antitumor potential of various mushroom species. For example, Hu et al. [28] examined the antitumor effect of MEP-II polysaccharide from fermentation broth of *M. esculenta* and reported that this substance inhibited cell proliferation of HepG2 cell line through an apoptotic pathway. They treated the cells with 150-600 µg MEP-II/ml. Similarly, Nitha et al. [29] showed anti-tumor activities of ethanolic extract from mycelium of *M. esculenta* against both ascites and solid tumors. In addition, antitumor activity of phenolic compounds from *M. esculenta* were listed in the review by Ajmal et al. [30].

Anticancer activity of ethanolic extract from an edible mushroom *R. flava* was evaluated by Liu et al. [20]. They reported that it showed strong cytotoxicity (71.6%) against a tumor cell line MDA-MB-231 at 200 µg/ml concentration. Similarly, we found methanolic extract of *R. flava* was one of the most effective mushrooms on cytotoxicity of A-549 tumor cell line and it showed 67% cytotoxicity at 400 µg/ml. This suggest that *R. flava* has potential as natural antitumor substance and effective on different cancer cell lines.

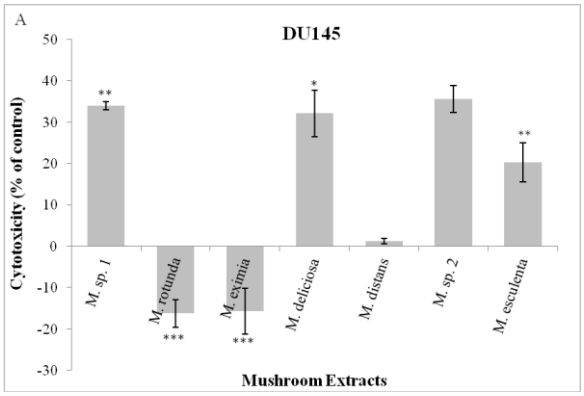
Antitumor capacity of benzoate derivatives and hirsutane type sesquiterpenoids obtained from solid state fermented rice with *Stereum hirsutum* was investigated on A549 and HepG2 cell lines [19]. Even though cytotoxicity of benzoate derivative on A549 cells was much stronger than its activity on HepG2 cells, sesquiterpenoids was more effective on the cytotoxicity of HepG2 cells. IC_{50} values of these compounds changed between 10-50 µM. In this present study, cytotoxic effect of methanolic extract from *S. hirsutum* was found to be 47% on A-549 cells. However, comparision of the results from these two studies may not be suitable because we used crude methanolic extract whereas they used isolated compounds from ethanolic extracts of fermented rice substrate with *S. hirsutum.*

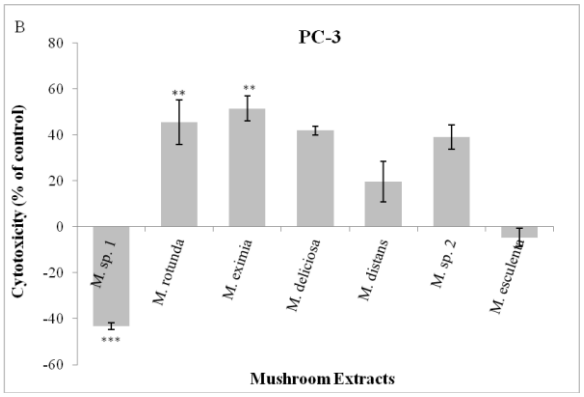
When antitumor activity of *C. cibarius* methanolic extract was examined in this present study, different levels of cytotoxicity 55, 62 and 52% were detected on A-549, CCC-221 and PC-3 cell lines, respectively. Kozarski et al. [24] reported selective cytotoxic effect of methanolic extract of *C. cibarus* on HeLa, MDA-MB-453, K562 cell lines, compared to normal cell line BEAS-2B. Similar to their findings, we also found that cytotoxic effect of *C. cibarus* extract was selective on cancer cell lines K562, DU-145, PC-3 and CCC-221 versus normal cell line BEAS-2B. For example, cytotoxicity was twofold higher on K562 cells compared to BEAS-2B cell line which is less sensitive to cytotoxic action of the extract (data not shown).

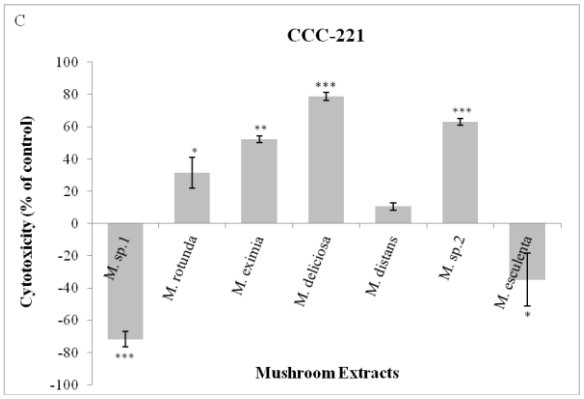
In short, antibacterial and antitumor actions of most of the macrofungi extracts examined in this study are similar or different from those reported in the literature. It has been known that different geographical locations and climatic conditions cause the difference in seconder metabolites of plants. Similarly, chemical compositon of *C. cibarius* extract is effected by a number of factors such as macrofungi strain, nutritional value of the growth environment, time of harvest, handling conditions and preperation techniques [31]. In addition, levels and and quality of phytochemicals from mushroom species have been signifcantly changed depending on stress conditions such as UV radiation, air pollution, parasites, wounding, infection by pathogens and exposure to extreme

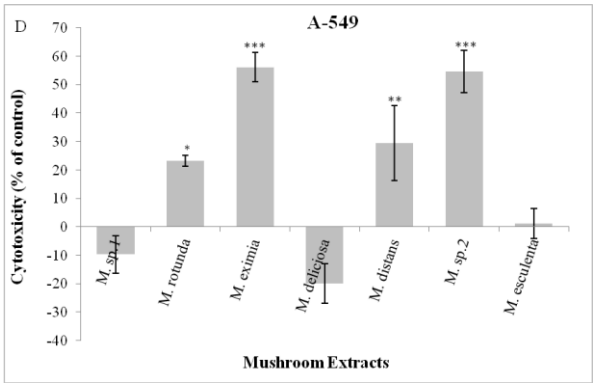
temperatures [32, 33]. Therefore, it is important to search antibacterial and antitumor potentials of mushrooms belonging to the same taxa from different geographycal regions.

In conclusion, antibacterial and antitumor activities of nine different mushroom species and seven different *Morchella* species were investigated in this study. The highest antibacterial activity was observed with *M. excissa* extract against *E. coli*. Mushroom extracts exhibited cytotoxic effect on some cancer cell lines but some of them induced cell proliferation. The highest cytotoxic effect (79%) was observed with *M. deliciosa* extract on CCC-221 cell line and with *M. distants* extract (72%) on K562 cell line. In addition, *T. versicolor* was the most effective compared to other mushroom species and it caused 72% cytotoxicity on A-549 cell line. In a future study, antitumor components of effective mushroom extracts as well as mechanism of antitumor action will be clarified.









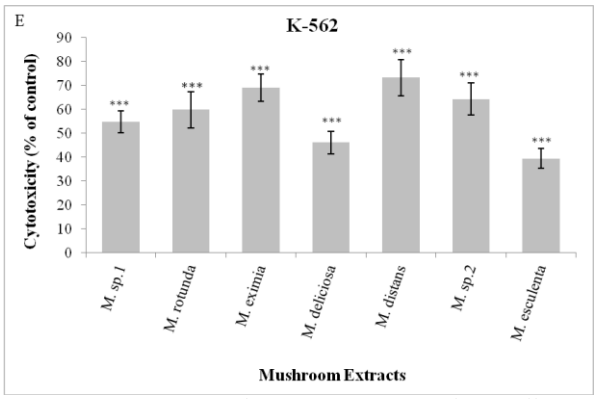
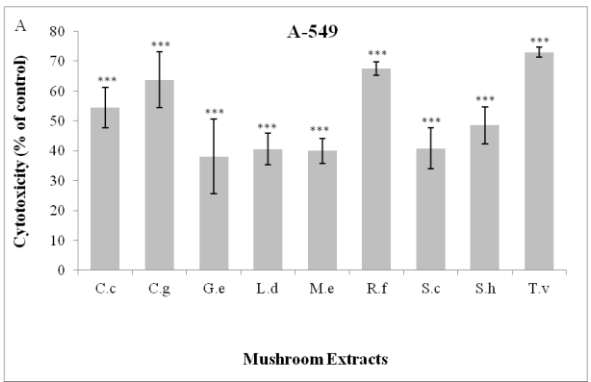


Figure 1. Cytotoxicity of mushroom extracts from different *Morchella* species on cell proliferation. The cells were plated onto 96-well plates and tretaed with or without (control) each mushroom extract (400 µg/ml) for 72 hr. Data are means (±SD) of three replicates. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.



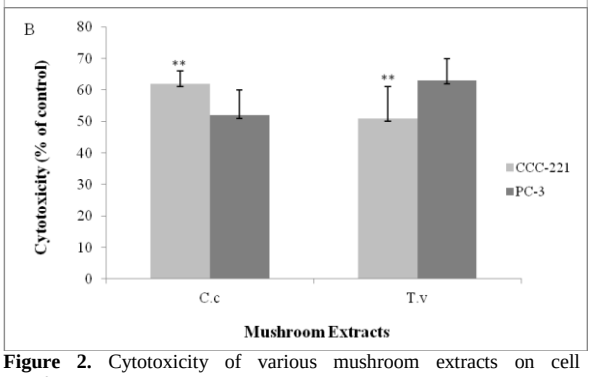


Figure 2. Cytotoxicity of various mushroom extracts on cell proliferation. The cells were plated onto 96-well plates and tretaed with or without (control) each mushroom extract (400 µg/ml) for 72 hr. Data are means (±SD) of three replicates. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$. **C.c**: *Cantharellus cibarius*; **C.g**: *Clitocybe geotropa*; **G.e**: *Gyromitra esculenta*; **L.d**: *Lactarius delicious*; **M.e**: *Melanoleuca exscissa*; **R.f**: *Ramaria flavescens*; **S.c**: *Sarcosphaera crassa*; **S.h**: *Stereum hirsitum*; **T.v**: *Trametes versicolor*

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