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ORIGINAL ARTICLE

Assessment of the antimicrobial, antioxidant and cytotoxic activities of the wild edible mushroom *Agaricus lanipes* (F.H. Møller & Jul. Schäff.) Hlaváček

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Abstract The present investigation was undertaken to evaluate the antimicrobial and antioxidant activities of the wild edible mushroom *Agaricus lanipes*, and also to investigate its cytotoxicity and potential and possible apoptotic effect against the A549 lung cancer cell line in in vitro conditions. Total antioxidant capacity, total phenolic content, total oxidant status, total antioxidant status, lipid hydroperoxides, and total free –SH levels of *A. lanipes* were found to be 4.55 mg T/g, 14.6 mg GA equivalent/g, 3.10 mg H_2O_2 equivalent/g, 2.25 mg H_2O_2 equivalent/g, and 1.90 μmol/g, respectively. The methanolic extract of *A. lanipes* had relatively strong antimicrobial activity against seven tested microorganism strains. It also had high antiproliferative potency and strong pro-apoptotic effects, and this mushroom used as a daily nutrient could be a source for new drug developments and treatment in cancer therapies, and could be a guide for studies in this area.

Keywords *Agaricus lanipes* · Antimicrobial · Antioxidant · Cytotoxic effect

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Introduction

The health benefits of regular consumption of fungi or bioactive compounds isolated from fungi and the use of fungi as functional food or nutraceutical product gain great importance, when it comes to the potential pharmacological properties of edible fungi such as their antioxidant, anticancer, antibiotic (Liu et al. 2013), antimicrobial (Alves et al. 2012), antiviral, antifungal (Wong et al. 2010), antitumor (Tong et al. 2009), hepatoprotective, antidiabetic (Wasser and Weis 1999), anti-inflammatory, antiatherogenic, antiallergic, immunomodulating, hypoglycemic, hepatoprotective (Lindequist et al. 2005) and cytotoxic (Guerra Dore et al. 2007) properties.

Nowadays predominant therapeutic methods such as chemotherapy and surgery are commonly used for cancer patients, but these methods have not been fully effective on many cancers. Alternative and complementary medicine is now very popular for many disorders and is used by patients who are undergoing or have completed medical treatment for various types

of cancer, and a combination of multi-therapeutic steps may effectively improve the treatment of cancer (Wu et al. 2012). Some edible medicinal mushrooms have been tested against several cancers such as breast cancer (Moongkardndi et al. 2004), prostate cancer (Gu and Sivam 2006), liver cancer (Menikpurage et al. 2012), colon cancer (Rosa et al. 2012), leukemia (Lin et al. 2012), lung cancer and gastric cancer (Vaz et al. 2010). Some research has focused on the antitumor activity and effect of the carbohydrate fractions of mushrooms. For instance, lectins isolated from mushrooms have been shown to have activities as chemotherapeutic drugs on HCT8 human colonic cancer cells, A549 human lung adenocarcinoma cells and T98G human glioblastoma cells (Guto et al. 2002), and to increase cellular antioxidant defense mechanisms (Shi et al. 2002). Despite all these researches with different mushroom species, there is no study about cytotoxic activity of *Agaricus lanipes.*

The aim of the present study was to evaluate the antimicrobial and antioxidant activities of the wild edible mushroom *A. lanipes* and to investigate its cytotoxicity and potential and possible apoptotic effect mechanism. To our knowledge, this is the first report on the antimicrobial, antioxidant and cytotoxic activities of *A. lanipes.*

Materials and methods

Macrofungal materials

Fruit bodies of *A. lanipes* samples were collected in the forest around Gireniz Valley, located in the western part of Denizli Province, Turkey in the course of field work in 2010 and 2013. *A. lanipes* is a fungus species in the family of Agaricaceae, and usually grows on neutral and calcareous soils in forests associated with *Pinus*. It is a wild edible mushroom gathered mostly in spring. Identification and classification of macrofungi were carried out at the laboratory of the Mushroom Research and Application Center (Pamukkale University, Denizli, Turkey).

Preparation of the extract

Fresh mushrooms were randomly divided into three samples of 75 g and then dried in an oven at 40 ± 0.1 °C before analysis. Approximately 25 g of the dried mushroom samples was weighed and then extracted by stirring with 250 ml of methanol (Merck, Darmstadt, Germany) at room temperature at 150 rpm for 24 h and filtered through filter paper (Whatman No. 4). The residue was then extracted with an additional two 250 ml of methanol as described above. The combined methanolic extracts were then evaporated at 40 ± 0.1 °C to obtain dry extract, re-dissolved in methanol to a concentration of 10 mg ml^{-1} and stored at 4 °C for further use.

Antioxidant activity

Determination of total antioxidant capacity

Total antioxidant capacity (TAC) was determined by using ABTS radical. Total antioxidant activity of the samples was determined according to a method described by Erel (2004), using commercially available kit (TAC kit, Relassay, Gaziantep, Turkey). ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), is a radical cation which remains stable for a long time in the acetate buffer solution. When it is diluted with a more concentrated acetate buffer solution at high pH values, the color is spontaneously and slowly bleached. Antioxidants present in the sample accelerate the bleaching rate to a degree proportional to their concentrations, which can be monitored spectrophotometrically, and the bleaching rate is inversely related with the TAC of the sample. The reaction rate is calibrated with Trolox, which is widely used as a traditional standard for TAC measurement assays. All reagents were purchased from Sigma-Aldrich (Interlab A.S., Istanbul, Turkey) unless otherwise specified. The results were expressed as mg Trolox equivalent/g.

Total phenolic content analysis

Total phenolic content (TPC) was determined according to the modified method of Skerget et al. (2005) that is based on a colorimetric oxidation/reduction reaction. For this purpose 9 ml of working solution (methanol/water:1/1) was added to 1 g sample and this mixture was kept at 4 °C for 24 h by shaking periodically, then centrifuged for 5 min at 5000 rpm. 2 ml of the supernatant was taken into a tube and mixed with 10 ml of tenfold diluted Folin–Ciocalteu reagent (Sigma-Aldrich, Interlab A.S.). 8 ml of

sodium bicarbonate solution (7.5% w/w, Merck) was added to the mixture and incubated at room temperature for 2 h, after which absorbance was read at 760 nm in a spectrophotometer (Shimadzu UV-1601, Shimadzu Scientific Instruments, Inc., Tokyo, Japan) against blank. The working solution used was blank and gallic acid (Sigma-Aldrich) in standard preparation. Results were expressed as mg gallic acid equivalent (GAE)/g.

Measurement of lipid hydroperoxides

Oxidation of Fe II to Fe III by lipid hydroperoxides (LOOHs) under acidic conditions was followed by complexation of Fe III by xylenol orange. This was carried out in a spectrophotometer (Shimadzu UV-1601, Shimadzu Scientific Instruments, Inc.) with two reagents, in a two-end-point mode with bichromatic detection at 570 and 700 nm (Arab and Steghens 2004). All reagents were purchased from Sigma-Aldrich (Interlab A.S.) unless otherwise specified. The results were expressed as mg H_2O_2 equivalent/g.

Measurement of total free sulfhydryl groups

Total free sulfhydryl (TFS) groups in the samples were assayed according to the method of Elman (1959). Briefly, 2 ml of buffer containing 0.1 M Tris, 10 mM EDTA (pH 8.2), and 100 µl sample was added to cuvettes, followed by 100 µl 10 mM DTNB in methanol. Blanks were run for each sample as a test, but there was no DTNB in the methanol. Following incubation for 15 min at room temperature, sample absorbance was read at 412 nm on spectrophotometer (Shimadzu UV-1601, Shimadzu Scientific Instruments, Inc.). Sample and reagent blanks were subtracted. The concentration of sulfhydryl groups was calculated using reduced glutathione as free sulfhydryl group standard and the result was expressed as µmol/g. All reagents were purchased from Sigma-Aldrich (München, Germany) unless otherwise specified.

Determination of total oxidant status

Total oxidant status (TOS) of mushroom was determined according to Erel (2005) by using a commercial kit (TOS kit, Relassay). Oxidants present in the sample oxidize the ferrous ion-o-dianisidine complex to ferric ions. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric ion makes a coloured complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay is calibrated with hydrogen peroxide and the results are expressed in terms of milligrams hydrogen peroxide equivalent (mg H_2O_2 equivalent/g).

Antimicrobial activity

Microorganisms

Crude methanol extracts of the fruiting bodies of *A. lanipes* were screened in vitro for antimicrobial activities against three Gram-positive bacteria (*Micrococcus luteus* NCIBM, *Staphylococcus aureus* ATCC 25923, and *Bacillus subtilis* ATCC 6633); three Gram-negative bacteria (*Proteus vulgaris* RSKK 96026, *Escherichia coli* ATCC 35218, and *Yersinia enterecolitica* RSKK 1501) and against one human fungal pathogen (*Candida albicans* ATCC 10231). The bacteria and the fungus were obtained from the culture collection of the Food Engineering Department of Pamukkale University. Bacterial cultures and *C. albicans* were maintained on Müller-Hinton agar substrates.

Screening of antimicrobial activity of the mushroom extract

Antimicrobial activity of methanolic extract of *A. lanipes* was determined by the disc diffusion method (Collins and Lyne 1987). Bacterial strains and *C. albicans* were cultivated on Mueller–Hinton Broth (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA). A suspension of the microorganism strain to be tested was spread on solid media plates. Then 6 mm sterilized discs (Sigma-Aldrich, Interlab A.S.) were soaked in 50 µl of the pure extract and placed on the inoculated plates, and kept at 4 °C for 2 h, after which they were incubated at 37 ± 0.1 °C for 18–24 h for bacteria. At the end of the incubation period, the diameters which the inhibition zones formed on the medium were evaluated in mm. All assays were carried out in duplicate and the inhibition zones were compared with those of the reference discs. Ampicillin (10 µg), Gentamicin (10 µg), Tetracycline (30 µg)

and Oxacillin (1 μg) was used as a positive control. All the antibiotics were provided from Sigma-Aldrich (Interlab A.S.).

Anti-proliferation and cytotoxic activity

Tumor cell line

A549 human lung adenocarcinoma cell line (obtained from ATCC, Manassas, VA, USA) was used in this study.

Cell culture and chemicals

A549 cells were grown in DMEM medium supplemented with 2 mM L-glutamine, penicillin (20 units/ml), streptomycin (20 μg/ml), and 10% (v/v) heat-inactivated fetal calf serum at 37 °C in a saturated humidity atmosphere containing 95% air and 5% CO_2. A549 cells were treated with the extract at concentrations of 25, 100, 200, 250, 400, 750 μg/ml, and 1 or 2 mg/ml by dissolving in the culture medium for 72 h, in a time and dose dependent manner. The following chemicals were purchased from Biological Industries (Kibbutz, Beit Haemek, Israel): DMEM medium, penicillin/streptomycin mixture, phosphate buffered saline (PBS), heat inactivated fetal bovine serum (FBS) and XTT assay kit. Transcriptor first strand cDNA synthesis kit was purchased from Roche Diagnostics (Mannheim, Germany). Ethidium bromide solution was provided from Applichem (Darmstadt, Germany). Trypan blue solution and Agarose were obtained from Sigma-Aldrich (Interlab A.S.).

Cytotoxicity assay

Cytotoxicity assays and determination of IC_{50} dose of extract in A549 cells were performed by using trypan blue dye exclusion test and XTT (Biological Industries) assay as indicated in the manufacturer's instructions.

XTT assay Cells were seeded in 96-well tissue culture plates and incubated for 24 h without reagent. After addition of reagents, cells were incubated for 24, 48 and 72 h and cell viability was assessed by using XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) mixture as recommended by the supplier. Formazan formation was quantified spectrophotometrically at 450 nm (reference wavelength was 630 nm) using a microplate reader. Viability was calculated using the background corrected absorbance as follows:

$$\text{Viability}\,(\%) = \text{A of experiment well/A of control well} \times 100.$$

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated from the cells exposed to IC_{50} doses of extract with Tri-Reagent (Invitrogen; Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis was performed using First-Strand cDNA Synthesis Kit (Roche Diagnostics). Appropriate cycles were chosen to ensure the termination of PCR amplification.

Semi-quantitative PCR (Techne, Burlington, NJ, USA) products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining and photographed under ultraviolet light. In this study, *Cyclin D1* (*CCND1*), *Bax*, *Bcl-2*, *Caspase 3*, *Caspase-9*, *p21*, *p53*, and *Rel A* (*p65*) gene expressions were analyzed in the control and dose group and GAPDH was used as a housekeeping gene.

Table 1 Primer sequences of the genes used in this study

Name	Primer sequence
CCND1	F: AGCTCCTGTGCTGCGAAGTGGAAAC
	R: AGTGTTCAATGAAATCGTGCGGGGT
Rel A	F: AGCAGCGTGGGGACTACGAC
	R: AGGCTGGGGTCTGCGTAGGG
BAX	F: AGAGGATGATTGCCGCCGT
	R: CAACCACCCTGGTCTTGGATC
CASPASE-3	F: GCAGCAAACCTCAGGGAAAC
	R: TGTCGGCATACTGTTTCAGCA
BCL-2	F: TTGGCCCCCGTTGCTT
	R: CGGTTATCGTACCCCGTTCTC
GAPDH	F: TTCTATAAATTGAGCCCGCAGCC
	R: CCGTTGACTCCGACCTTCAC
CASPASE-9	F: GGCTGTCTACGGCACAGATGGA
	R: CTGGCTCGGGGTTACTGCCAGF:
P21	TGGAGACTCTCAGGGTCGAAA
	R: GGCGTTGGAGTGGTAGAAATC
P53	F: ATCTACAAGCAGTCACAGCACAT
	R: GTGGTACAGTCAGAGCCAACC

Primer sequences are given in Table 1. The mRNA expression image bands were quantified using Socion Image Version Beta 4.0.2 software and were reported relative to *GAPDH* mRNA level.

Results and discussion

Antioxidant status

The results obtained for the methanolic extract of total antioxidant capacity, total phenolic content, total oxidant status, total antioxidant status, lipid hydroperoxides, and total free –SH levels from *A. lanipes* are given in Table 2.

Total antioxidant capacity

Because of the chemical structure of the phenolic compounds found in edible fungi, they both have powerful antioxidant effects and can be used as an important indicator of antioxidant capacity. Various researchers have shown a close relationship between antioxidant activity and content of phenolic matter (Kyslychenko et al. 2010). Continual exposure to chemicals, stress and pollutants causing an increase in free radicals can cause oxidative damage to biomolecules such as carbohydrates, lipids, proteins and DNA in humans. Researchers have shown that the consumption of foods with high phenolic matter content can lower the risk of heart attack by slowing the advance of atherosclerosis and that including up to 1.0 g in the daily diet can have the effect of halting mutagenesis and carcinogenesis (Tanaka et al. 1998). In addition, antioxidants and certain phenolic compounds can reduce the risk of diabetes, aging and other degenerative diseases in humans (Halliwell 1996). In the present study, the total antioxidant capacity of *A. lanipes* was found to be 4.55 mg Trolox equivalent/g (Table 2). In a study by Robaszkiewicz et al. (2010), it was reported that the antioxidant capacity values of methanolic extracts of various species of *Agaricus*, *Boletus*, *Suillus*, *Tricholoma* and *Tuber* varied between 0.11 and 5.14 mg Trolox equivalent/g. These data show that the methanolic extract of *A. lanipes* has a high level of antioxidant capacity.

Total phenolics content

Total phenol acid and monophenolic and polyphenolic substances are found naturally in some fungi and these are related to antioxidant activity (Puttaraju et al. 2006). Total polyphenols were the major naturally occurring antioxidant components found as 14.6 mg GAE/g in methanolic extracts from *A. lanipes*. Woldegiorgis et al. (2014) determined the total phenolics in extracts of *A. campestris* to be 14.6 mg GAE/g. Barros et al. (2008) reported the total phenol contents of extracts of *Agaricus bisporus*, *A. arvensis*, *A. romagnesii*, *A. silvaticus* and *A. silvicola* as 4.49, 2.72, 6.18, 8.95 and 6.40 mg/g respectively, and reported the most important antioxidant compounds in the extracts of these fungi to be phenolics.

Measurement of lipid hydroperoxides

Phenolic compounds are associated not only with antioxidant activity but also play an important role in stabilizing lipid peroxidation (Yen et al. 1993). We used iron to measure lipid hydroxyperoxides in our study because it can stimulate lipid peroxidation by the Fenton reaction, and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Chang et al. 2002). It was reported that lipid hydroperoxides appear to be good candidates as initial biomarkers of oxidative stress; they are a large family of the first by-products of oxidized lipids, and their quantification could become a useful biomarker (Shi et al. 2002). The lipid hydroperoxide content of *A. lanipes* was determined as 2.25 mg H_2O_2 equivalent/

Table 2 Antioxidant status of the methanolic extract of *A. lanipes*

Sample	TAC	TPC	LOOHs	TFS	TOS
A. lanipes	4.55 ± 0.62	14.6 ± 1.02	2.25 ± 0.14	1.90 ± 0.02	3.10 ± 0.61

TAC Total antioxidant capacity, mg Trolox equivalent/g; *TPC* total phenolics content, mg GAE equivalent/g; *LOOHs* measurement of lipid hydroperoxides, mg H_2O_2 equivalent/g; *TFS* total free sulfhydryl, μmol/g; *TOS* total oxidant status, mg H_2O_2 equivalent/g

g. In a study by Barros et al. (2008), it was reported that the species *A. bisporus*, *A. arvensis*, *A. romagnesii*, *A. silvaticus* and *A. silvicola* of the Agaricaceae family had lipid peroxidation inhibition capacity.

Total free sulfhydryl group

When an organism is exposed to oxidative stress, –SH groups are among the first antioxidants to be consumed. Free sulfhydryl (–SH) groups of proteins are mainly responsible for their antioxidant response, and they can serve as a sensitive indicator of oxidative stress (Gu and Sivam 2006). The total free sulfhydryl content of *A. lanipes* extract in our study was found to be 1.90 μmol/g. Thiols are very susceptible to oxidation and sulfhydryl groups are known to scavenge aqueous peroxyl radicals; they are considered to be one of the most important plasma sacrificial antioxidants (Wayner et al. 1987).

Total oxidant status

Total oxidant status also serve significantly important information for understanding the antioxidant-oxidant balance of the mushroom. Total oxidant status was found in this study to be 3.10 mg H_2O_2 equivalent/g.

Antimicrobial activity

The antimicrobial activity of a methanolic extract obtained from the fruiting organ of *A. lanipes* was tested against three Gram-positive bacteria, three Gram-negative bacteria and one species of yeast. As is summarized in Table 3, methanolic extract of *A. lanipes* at the test concentration showed an antimicrobial effect against all the test microorganism strains, forming inhibition zones of 11–22 mm. Inhibition zone values obtained in the study were greatest for the bacterium *M. luteus* NCIBM (22 ± 1 mm, inhibition zone diameter), which showed the highest sensitivity to the extract, and least for the bacterium *E. coli* ATCC 35218 (11 ± 0 mm, inhibition zone diameter), which showed the lowest sensitivity. Alongside the bacterial species, it was shown that the yeast species *C. albicans* ATCC 10231 was sensitive to the extract of *A. lanipes* (19 ± 1 mm, inhibition zone diameter). While, only some of the commercial antibiotics used in our study showed an antimicrobial effect, the *A. lanipes* extract was observed to have an antimicrobial effect against all the test microorganism strains.

Various extracts of the species *A. bisporus*, *A. bitorquis*, *A. essettei*, *A. silvicola* and *A. brasiliensis* from the Agaricaceae family have shown antimicrobial effects against various microorganisms such as *M. luteus*, *S. aureus*, *B. subtilis*, *P. vulgaris*, *Y. enterecolitica* and *C. albicans*; however, the *A. lanipes* extract in our study additionally showed an antimicrobial effect, albeit a little one, against the bacterium *E. coli* (Table 4). Although the same test concentrations of the gram-positive and gram-negative bacteria and the yeast species were used, it is thought that the results indicating differences in sensitivity may arise from varying methodologies and from differences in the permeability of cell walls in the microorganisms. The results are promising for the use of *A. lanipes* as an antimicrobial agent.

Table 3 Antimicrobial activity of the extracts of *A. lanipes* against bacterial strains and *C. albicans* (diameter of inhibition zones, mm)

Bacteria	*A. lanipes*	A	O	G	T
Micrococcus luteus NCIBM	22 ± 1	30	22	na	19
Staphylococcus aureus ATCC 25923	13 ± 1	na	21	na	20
Bacillus subtilis ATCC 6633	20 ± 0	na	8	na	17
Proteus vulgaris RSKK 96026	15 ± 0	–	na	na	16
Escherichia coli ATCC 35218	11 ± 0	10	na	na	8
Yersinia enterecolitica RSKK 1501	17 ± 1	20	na	na	7
Candida albicans ATCC 10231	19 ± 1	na	na	na	na

A Ampicillin (10 μg), *O* Oxacillin (1 μg), *G* Gentamicin (10 μg), *T* Tetracycline (30 μg), *na* not active, – no inhibition

Table 4 Antimicrobial effects of *Agaricus* species against various microorganisms

Fungus	Microorganism	References
Agaricus bisporus	*Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus vulgaris*, *Yersinia enterecolitica*, *Candida albicans*	Ozturk et al. (2011), Alves et al. (2012)
Agaricus bitorquis	*Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus vulgaris*, *Yersinia enterecolitica*, *Candida albicans*	Ozturk et al. (2011), Alves et al. (2012)
Agaricus essettei	*Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans*	Ozturk et al. (2011)
Agaricus silvicola	*Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*	Alves et al. (2012)
Agaricus brasiliensis	*Staphylococcus aureus*, *Bacillus cereus*, *Candida albicans*	Mazzutti et al. (2012)
Agaricus lanipes	*Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus vulgaris*, *Escherichia coli*, *Yersinia enterecolitica*, *Candida albicans*	In this study

Antiproliferation and cytotoxic activity

Cytotoxic activity

XTT assay A549 cell death on treatment with extract of *A. lanipes* was assessed using the XTT assay. Time- and dose-dependent decrease patterns were found in the viability of A549 cells. For this purpose, the expression changes of *CCND1*, *Bax*, *Bcl-2*, *caspase-3*, *caspase-9*, *p21*, *p53*, *Rel A* (*p65*) genes were evaluated at the 24th, 48th and 72nd h after treating A549 cells with different concentration of the extract. In our study, an IC_{50} dose (inhibitory concentration where 50% of the cells die) in the A549 cells was detected as 250 µg/ml at the 72nd h by XTT assay (Fig. 1).

Semi-quantitative RT-PCR

We determined the expression of selected genes in cells exposed to the extract. Using the semi-quantitative PCR method, mRNA levels were analyzed in extract-treated cells and these were compared with the levels of mRNA in untreated cells.

Expression results showed that *Bcl-2* and *CCND1* gene expression levels in A549 cells were decreased in dose group cells compared to the control cells in a time-dependent manner. While *Bax*, *caspase-3*, and *p65* gene expression was increased in the dose group (Figs. 2, 3), there were no expression changes in other genes compared with the control cells.

Although reports have shown that mushrooms have anticancer and anti-proliferative effects, there is no available research about cytotoxic effect of *A. lanipes* on the A549 cell line. To determine the possibility of using *A. lanipes*, its cytotoxic effects, cell viability and anti-proliferative activity were analyzed by using XTT assay. Our results showed that mushroom extract reduced the proliferation of A549 cells in a dose dependent manner compared to the untreated control cells.

Recent studies on the other species of the genus *Agaricus* have shown that mushrooms extracts are effective agents at various concentrations against different cancer cell types such as oral cancer (Fan et al. 2011), hepatocellular carcinoma (Su et al. 2011), osteosarcoma (Wu et al. 2012) and colon cancer (Wu et al. 2011). The anticancer mechanism of *A. lanipes* is still unclear, but there are many reports on mechanisms of other mushrooms. It was also found that mushroom extracts may affect the expression of genes of the anti- and pro-apoptotic super-family. The modulation of the expression of these genes is thought to play a role in sensitizing cancer cells to apoptosis. Su et al. (2011) reported that Blazeispirol A from *Agaricus blazei* fermentation product induces cell death in human hepatoma Hep3B cells through caspase-dependence by stimulating *caspase-9*, *caspase-3* and *PARP* and caspase-independent pathways by regulating *Bcl-2* family proteins. Another study indicated that a novel medicinal mushroom blend which including *A. blazei* suppresses the growth and invasiveness of MDA-MB-231 human breast cancer cells by inhibiting the expression of the cell cycle regulatory genes *ANAPC2*, *BIRC5*, *Cyclin B1*, *Cyclin H*, *CDC20*, *CDK2*, *CKS1B*, *Cullin 1*, *E2F1*, *KPNA2*, *PKMYT1* and *TFDP1* (Jiahua and Sliva 2010). Cheung et al. (2012) have shown that lectin from *Agaricus bisporus* inhibited S phase cell population and *Akt*

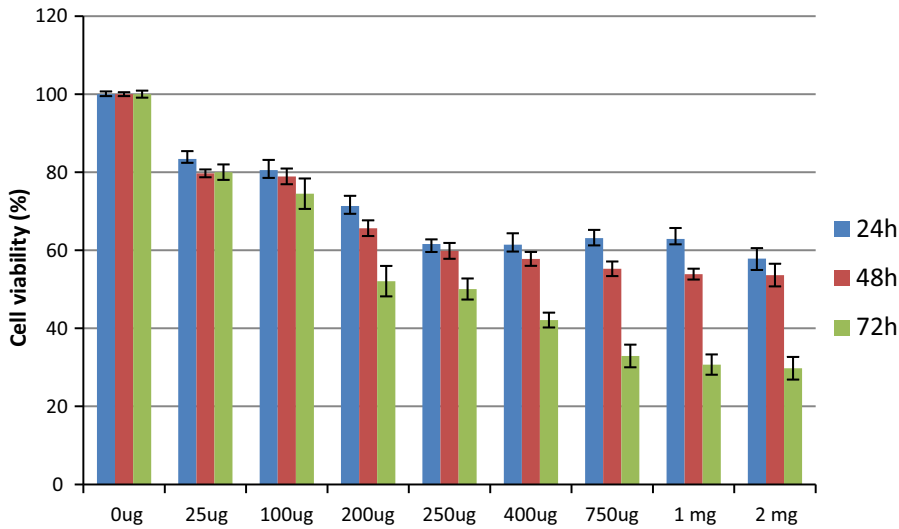

Fig. 1 Percentage cell viability of A549 cells induced by *A. lanipes* extract in different concentrations in XTT assay

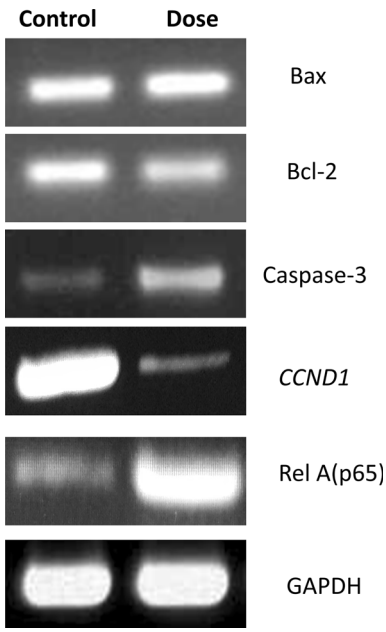

Fig. 2 mRNA expression changes in control and dose groups

phosphorylation in human RPE cells, and they suggested that *A. bisporus* arrested cell cycle progression was independent of *p53* activation. In an other research on a polysaccharide isolated from *A. blazei* Murill, it was reported that the mushroom has an anti-metastatic effect on BEL-7402 hepatic cancer cells and animal tumor models in in vitro and in vivo conditions by down-regulating *Metalloproteinase-9* and up-regulating *Nm23-H1* (Niu et al. 2009).

In the present study, we demonstrated the mechanism of action of *A. lanipes* extract by analyzing the expression of *CCND1*, *Bax*, *Bcl-2*, *caspase-3*, *caspase-9*, *p65 (Rel-A)*, *p53* and *p21* in the A549 lung cancer cell line. The modulation of the expression of these genes in thought to play a role in sensitizing cancer cells to apoptosis.

CCND1 is an important key regulator protein of the G1-S phase in the cell cycle, and Cyclin-CDK complexes have an important and consecutive role in the cell cycle, activating oncogenes and inhibiting tumor suppressors. *CCND1* promotes tumor growth in various cancer types by being over-expressed or amplified. *CCND1* has been also observed in many cancer types. p21 is a cyclin dependent kinase inhibitor and has a responsibility in cell cycle arrest as a tumor suppressor gene. *CCND1* gene expression level decreased in dose group cells compared to the control group. However, *p21* gene expression level was unchanged. *Bax*, *Bcl-2* and *caspase-3* and *caspase-9* are important members of apoptotic pathway in the cells. Our research showed that *Bcl-2* mRNA expression decreased, and *Bax* and *caspase-3* increased. Additionally, many studies have suggested that transcription factors of the NFκB family (Rel family) may control apoptosis in cancers and affect different members of apoptosis and cell survival. Our research results showed that p65 (Rel A) expression increased. According to the results, we suggest that *A. lanipes* extract can suppress cell cycle by down-regulating *CCND1* and induce apoptosis by

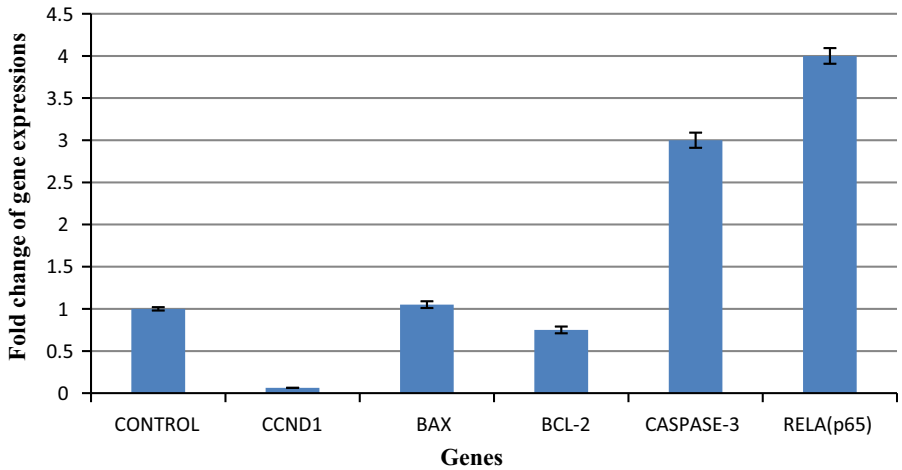

Fig. 3 The mRNA expressions of *CCND1*, *Bax*, *Bcl-2*, *Caspase-3* and *Rel A (p65)* genes relative to *GAPDH* mRNA expression were studied using Semi-quantitative RT-PCR. The mRNA expression image bands were quantified using Socion Image Version Beta 4.0.2 software and were reported relative to GAPDH mRNA level

modulating *Bax*, *Bcl-2* and *caspases* expression in A549 lung adenocarcinoma cells.

Conclusion

To our knowledge, this is the first report on the antimicrobial, antioxidant and cytotoxic activities of *A. lanipes*. As a result of the study, the total antioxidant capacity of *A. lanipes* was 4.55 mg Trolox equivalent/g, and total polyphenols were found to be 14.6 mg GAE equivalent/g. Lipid hydroperoxides content was determined to be 2.25 mg H_2O_2 equivalent/g, while total free sulfhydryl content was 1.90 µmol/g. Total oxidant status was found to be 3.10 mg H_2O_2 equivalent/g. In addition, methanol extract of *A. lanipes* was found to show an antimicrobial effect against all of the test microorganism strains.

In conclusion, *A. lanipes* extract had high antiproliferative potency and strong pro-apoptotic effects. The apoptotic mechanisms of *A. lanipes* extract are still not elucidated. There was no change in expression of *p53*, *caspase-9*, or *p21*. Other mechanisms such as the induction of apoptosis by receptor mechanisms or activating other pathways can modulate apoptosis. Identification of the active compounds is required for a better understanding of the protective mechanisms involved and for possible application in medicine.

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