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Effect of *Pleurotus ferulae* Extracts on Viability of Human Lung Cancer and Cervical Cancer Cell Lines

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Abstract When SiHa cells were incubated for varying periods of time with extracts of PFF and PFM, the cytotoxicity of the ethanol extracts of PFF was higher than those of the other extracts. These results indicated that the extracts from fruiting bodies of *P. ferulae* contain antitumor substances. When A549, SiHa and HeLa cells were incubated with different concentrations of PFF and PFM extracts, the ethanol extracts of PFF showed strong cytotoxicity against A549 cells at concentrations over 10 μg/mL and against SiHa and HeLa cells at concentrations over 40 μg/mL. However, the differences in the cytotoxic effects of the hot water and ethanol extracts of PFM and the hot water extracts of PFF on all 3 cancer cells were not significant. Also, the PFF ethanol extracts induced synergistic effects on the TRAIL-induced apoptosis in A549 cells, which were strongly resistant to TRAIL. These results indicated that ethanol extracts of PFF were the most prominent antitumor agents toward lung cancer cells (A549).

Keywords: *Pleurotus ferulae*, human lung cancer, cervical cancer cell line

INTRODUCTION

In recent years, there has been a heightened awareness of antitumor materials in the fast-growing field of biotechnology. For millennia, mushrooms have been valued by human kind as an edible and medical resource. A number of bioactive molecules, including antitumor substances, have been identified in many mushroom species [1-4]. Also, mushrooms are considered to be a good source of digestible proteins, with protein content above that of most vegetables, but somewhat less than most meats and milk. The protein content can vary from 10~40% on a dry basis. Mushrooms contain all the essential amino acids, but can be limited in the sulphur-containing amino acids, cystine and methionine [5]. Fresh mushrooms contain 3~21% carbohydrate and 3~35% fiber on a dry weight basis. Thus, a considerable proportion of the carbohydrate of mushrooms consists of dietary fiber, which can not be easily digested by humans, so function essentially as dietary fiber; in this way the calorific value of most mushrooms is low [6]. Polysaccharides are the best known and most potent mushroom-derived substances with antitumor and immunostimulating properties [7,8]. These polysaccharides have been identified as many types of glucans (*e.g.* β-1,3- and β-1,6-) [9,10]. Wide varieties of β-glucan applications have been reported, including thickening and stabilizing agents in chemical industries, and immunostimulating and antitumor agents in clinical uses [11,12].

Traditional medicines attribute their medicinal properties to *Pleurotus*, which is a well-known medicinal mushroom [13]. *Pleurotus* spp. are promising as medicinal mushrooms, exhibiting antibacterial [14], antiviral [15], anti-inflammatory [16], anticholesterolic [17], antitumor [18], and immunostimulating [19] activities. Although there have been many reports on the medicinal properties of different *Pleurotus* genus, much less is known about the effects of the biological activities of *Pleurotus ferulae*.

In this study, in order to investigate the on viability of human cancer cell lines for the screening of the antitumor substances contained in *Pleurotus ferulae*, the antitumor activities of these extracts were examined on three human solid carcinomas, a lung carcinoma (A549) and two cervical carcinoma (SiHa and HeLa).

MATERIALS AND METHODS

Cells and Culture

Human lung cancer (A549) and cervical cancer (SiHa, HeLa) cell lines were obtained from the Korean Cell Culture Bank and cultured in RPMI-1640 medium (GIBCO RBL, USA), supplemented with 10% (v/v) fetal bovine serum, 100 U/mL streptomycin and 100 U/mL penicillin Cells were maintained at 37°C in a humidified atmosphere, with 5% CO_2, and subcultured twice a week. Cells were incubated in a CO_2 incubator at 37°C in humidified atmosphere of 95% air and 5% CO_2 for varying periods of time, with or without *Pleurotus ferulae* extracts, and

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with different concentrations of *Pleurotus ferulae* extracts. The PFF and PFM materials obtained were washed four times with distilled water, dried in a drying oven at 60°C for 1 day and then powdered using a Wiley Mill with a 300 mesh particle size.

Preparation of Exo-polysaccharide

After the completion of the batch fermentation and centrifuging, all the supernatants were collected and the crude polysaccharide precipitated with the addition of 4 volumes of 98% ethanol. After standing the mixture overnight at 4°C, the precipitated exo-polysaccharide was collected by centrifugation, dissolved in distilled water, re-centrifuged to remove any insoluble materials, and lyophilized, the exo-polysaccharide obtained was used as the exo-polysaccharide of *P. ferulae*.

Extract Preparation of *Pleurotus ferulae*

About 200 g of dry powdered PFF and PFM were extracted with 1,000 mL of 95% ethanol, using a soxhlet apparatus at room temperature for 8~10 h. The extracts were evaporated in a rotary evaporator. The residue was dissolved in distilled water, the solvent evaporated and lyophilized, to give a solid mass with a yield of 3~5%, which was used as the ethanol extract of *P. ferulae*. About 100 g of dry powdered PFF and PFM were extracted with 1,000 mL of hot distilled water, using a soxhlet apparatus at 100°C for 2~3 h. After standing overnight at 4°C, the solvent was centrifuged, the supernatants evaporated and lyophilized, to give a solid mass with a yield of 12~13%, which was used as the hot water extract of *P. ferulae*.

Cytotoxicity Assay

The viability of the cells was determined by the MTT assay. The MTT assay is based on the optical measurement of a dye, formazan, produced from MTT by mitochondrial dehydrogenase. Human cancer cells were cultured on RPMI-1640, containing 10% FBS, with 2×10^4 cells per well added to 96-well microtiter plates. After the addition of various concentrations of *P. ferulae* extracts into each well, the 96-well plate was maintained in a CO_2 incubator (37°C) for 2 days. After the cultivation was complete, the RPMI-1640 was removed, and 50 μL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and 200 μL of fresh RPMI-1640 were added to the 96-well plate. Again, the plate was maintained in a CO_2 incubator for 4 h to allow the formazan formation. The quantity of formazan produced can be regarded as an indicator of the cell density or viability. After dissolving the formazan in 150 μL of DMSO (dimethyl sulfoxide), the absorbance at 540 nm was measured with a Microplate Autoreader (Labsystem Multiscan Multisoft, Finland). The results obtained were presented as a percentage of the control values. The control values were determined from cultures with cancer cells grown in the medium without any *Pleurotus ferulae* extracts, and were considered as 100%. Subsequently, the growth rates of the cancer cells treated with the hot water or ethanol extracts were calculated as the percentage of the control values and a graph plotted.

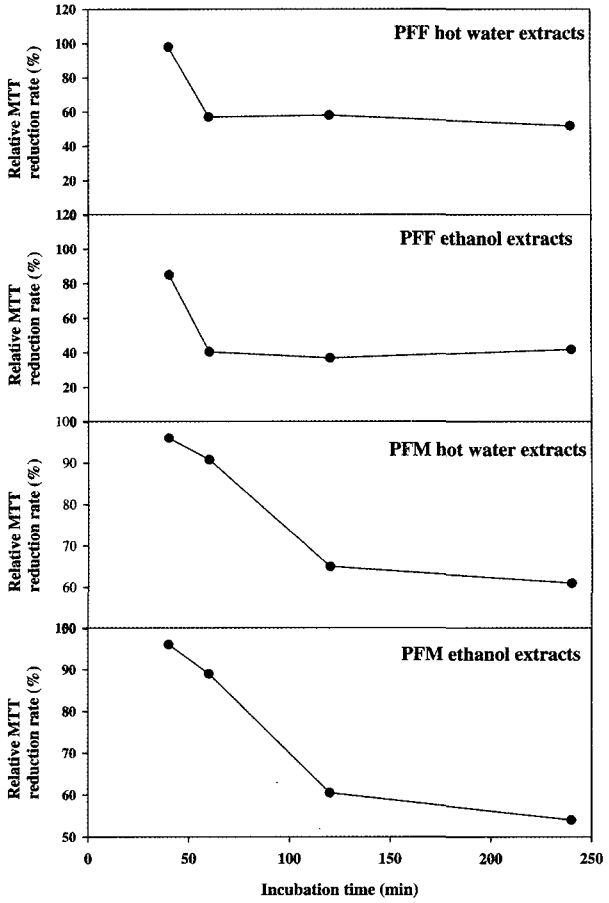

Fig. 1. Effect of incubation time of the PFF hot water extracts, PFF hot water extracts, PFM hot water extracts, and PFM ethanol extracts on the viability of SiHa cells. The control values were determined from cultures with cancer cells grown in the medium without any *Pleurotus ferulae* extracts, and were considered as 100%.

RESULTS AND DISCUSSION

The Cytotoxicity of *Pleurotus ferulae* Extracts on the SiHa

In order to investigate preliminary screening of the antitumor activity of the *Pleurotus ferulae* hot water and ethanol extracts, the cell growth of SiHa was measured with the MTT assay. The inhibitory effects of the PFF extracts, using hot water and ethanol extractions, on the SiHa growth are shown in Fig. 1. The hot water extracts of PFF was toxic to the cell growth, and the viable cells decreased to 49% after 120 min of incubation. Also, the ethanol extracts of PFF were significantly toxic to the

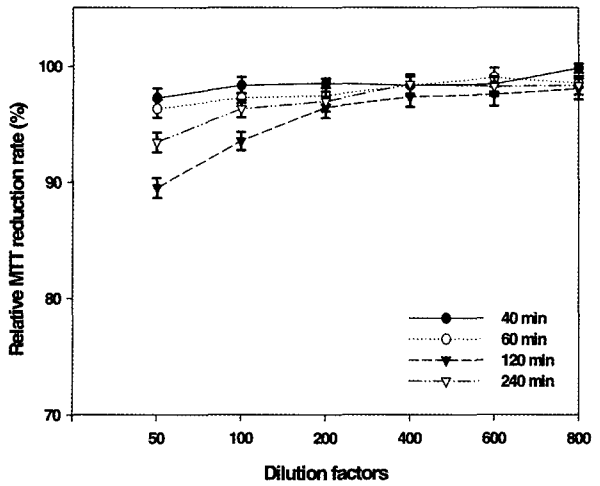

Fig. 2. Effect of incubation time of the exo-polysaccharide on the viability of SiHa cells. The cells were incubated with exo-polysaccharide, and the viability of cancer cells measured by the MTT reduction test.

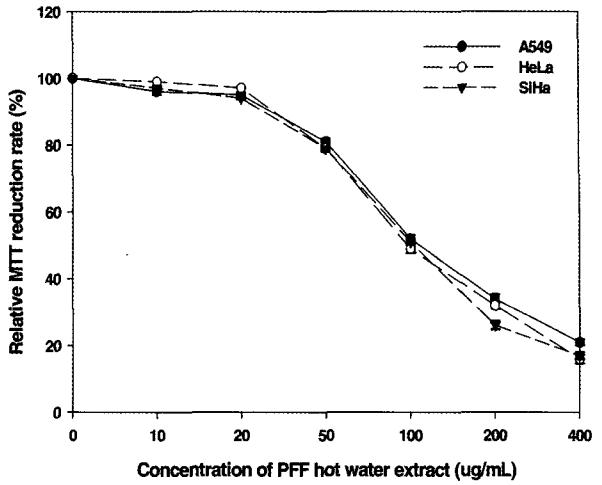

Fig. 3. Effect of PFF hot water extracts on the viability of human cancer cells. The cells were incubated with PFF hot water extracts for 2 days in a culture medium.

cells, and the viable cells decreased to 37% after 120 min of incubation. Both the hot water an ethanol extracts of PFF were significantly toxic to SiHa cells, but the cytotoxic effects of the ethanol extracts were more severe than the hot water extracts. The hot water extracts of PFM inhibited the growth of SiHa cells at a dilution factor of 50. The ethanol extract of PFM significantly inhibited the growth of SiHa cells at a dilution factor of 50.

At the dilution factor of 50, there is a difference in MTT rate.

Fig. 2 illustrates the inhibitory effect of exo-polysaccharide on the viability of SiHa cells. When SiHa cells were treated with exo-polysaccharide, no effect was observed. The reason the exo-polysaccharide did not inhibit the viability of SiHa cells was unclear, but it is evident more work will be required.

Toxicity of *Pleurotus ferulae* Extracts on Human Cancer Cell Lines

The effect of the *Pleurotus ferulae* extracts on the viability of cancer cells *in vitro* has been demonstrated. When the A549, SiHa and HeLa cells were incubated with 50 μg/mL of the hot water PFF extracts, the viabilities of the 3 cancer cells were measured by the MTT assay, and the results are shown in Fig. 3. The hot water extracts of PFF were found to be toxic to all three cancer cell lines. These results suggest that the hot water extracts of PFF have toxic effects on the cancer cells, and their toxicities were almost the same for all three cancer cells.

Fig. 4 illustrates the effects of the hot water PFM extract on the viability of the cancer cells *in vitro*. When the A549, SiHa and HeLa cells were cultured with 100 μg/mL of the hot water PFM extract, their viabilities, measured by MTT assay, were decreased. The inhibitory effects of the hot water extract of PFM were found to be

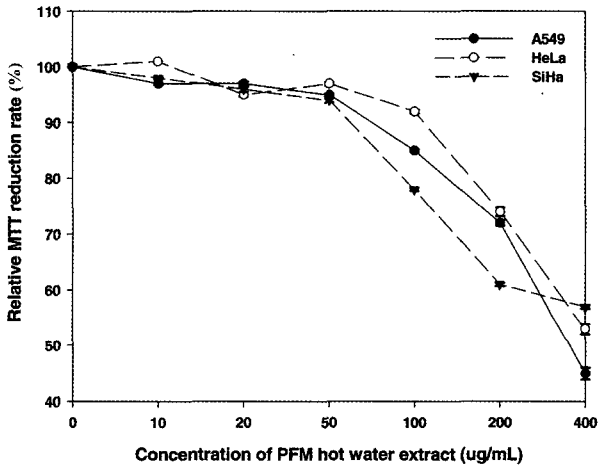

Fig. 4. Effect of PFM hot water extracts on the viability of human cancer cells. The cells were incubated with PFM hot water extracts for 2 days in a culture medium.

the same for all three cancer cells, but the cytotoxic activities were lower than the hot water extracts of PFF. When the A549, SiHa and HeLa cells were incubated with 40 μg/mL the PFF ethanol extracts, the viabilities of the SiHa and HeLa cells decreased, while the growth of A549 cells was inhibited at concentrations over 10 μg/mL (Fig. 5). These results suggest that the PFF ethanol extracts contain cytotoxic substances, with activities that are more potent than those of the PFM hot water extracts. Also, the A549 cells were the most sensitive to the cytotoxic effects of the PFF ethanol extracts.

The effects of the PFM ethanol extracts on the viabilities of the 3 cancer cells *in vitro* have been demonstrated, and are shown in Fig. 6. When the A549, SiHa and HeLa cells were incubated with 40 μg/mL of the PFM ethanol extracts, the viabilities of all three cancer cells were all

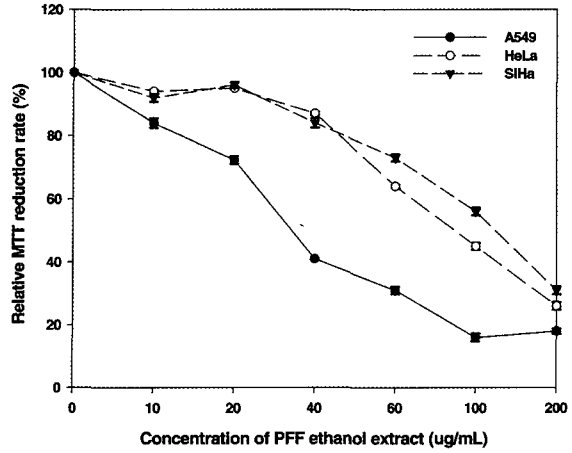

Fig. 5. Effect of PFF ethanol extracts on the viability of human cancer cells. The cells were incubated with PFF ethanol extracts for 2 days in a culture medium.

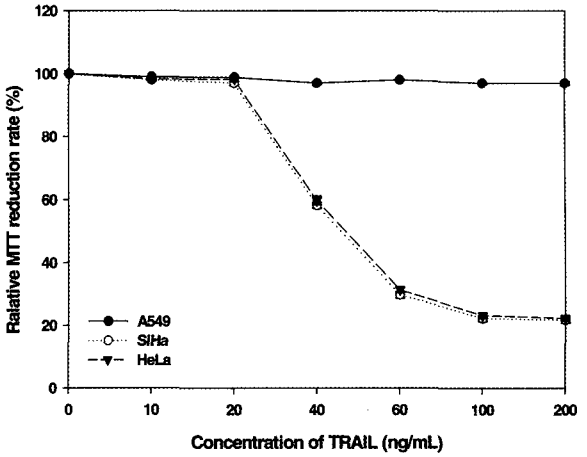

Fig. 7. TRAIL-induced apoptosis in A549, SiHa and HeLa cell lines. All three cancer cell lines were treated with various concentrations of TRAIL for 2 days.

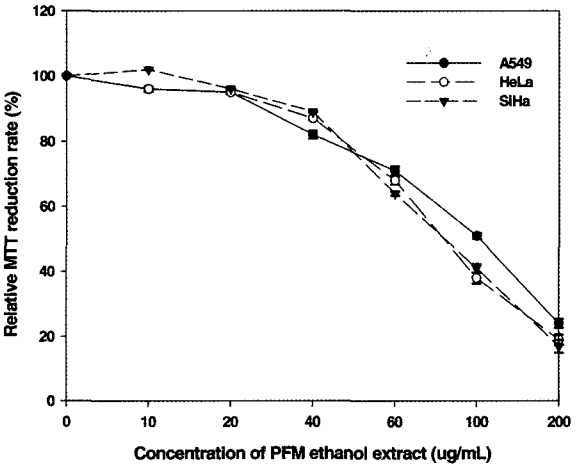

Fig. 6. Effect of PFM ethanol extracts on the viability of human cancer cells. The cells were incubated with PFM ethanol extracts for 2 days in a culture medium.

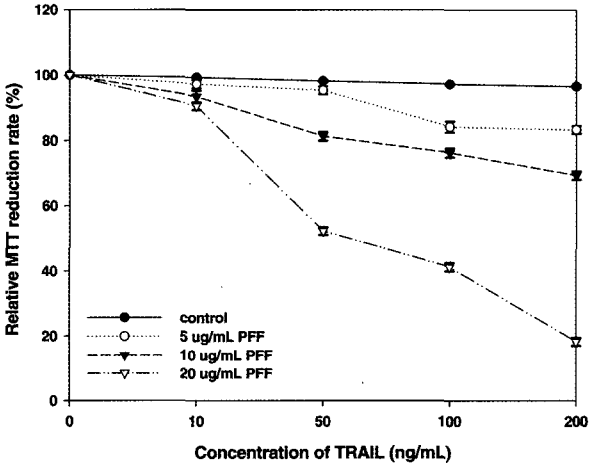

Fig. 8. Effect of PFF ethanol extracts on TRAIL-induced apoptosis in A549 cells. A549 cells were treated with various combinations of TRAIL and PFF ethanol extracts for 2 days.

decreased. The differences in the cytotoxic effects of the ethanol extracts of PFM for all three cancer cells were not significant. These results suggest that the cytotoxic effects of the ethanol extracts of PFM were almost the same for all three cancer cells. In conclusion, these results indicate that fruiting bodies of *Pleurotus ferulae* possess unknown kinds of antitumor agents.

Resistance to TRAIL and Synergistic Effect of PFF Ethanol Extracts Effect on TRAIL-induced Apoptosis in Human Cancer Cell Lines

TNF-related apoptosis-inducing ligand (TRAIL) is one of the apoptosis-inducing molecules of the tumor necrosis factor (TNF) family [20]. Apoptosis is a genetically controlled form of cell death, which appears to be involved in tumor cell killing by chemotherapeutic agents and cellular targeting [21]. To investigate the sensitivity to TRAIL-induced apoptosis in A549, SiHa and HeLa cell lines, three cancer cells were treated with various concentrations of TRAIL for 2 days, and the cell survivals assessed by MTT assays. The cervical cancer cell lines (SiHa, HeLa) were sensitive to TRAIL. When the A549 cells were exposed to TRAIL, no effect was observed (Fig. 7).

The cell survival of A549 cells treated with various combinations of TRAIL and PFF ethanol extracts for 2 days was assessed. When A549 cells were incubated with less than 20 μg/mL of PFF ethanol extracts, slight growth inhibition (less than 69.3%) was observed (Figs. 11 and 12). However, treatment with 20 μg/mL of PFF ethanol extracts plus 200 ng/mL TRAIL drastically inhibited the A549 cells, and the cell viable decreased to 18.3% (Figs. 8 and 9). The effect of PFF ethanol extracts on

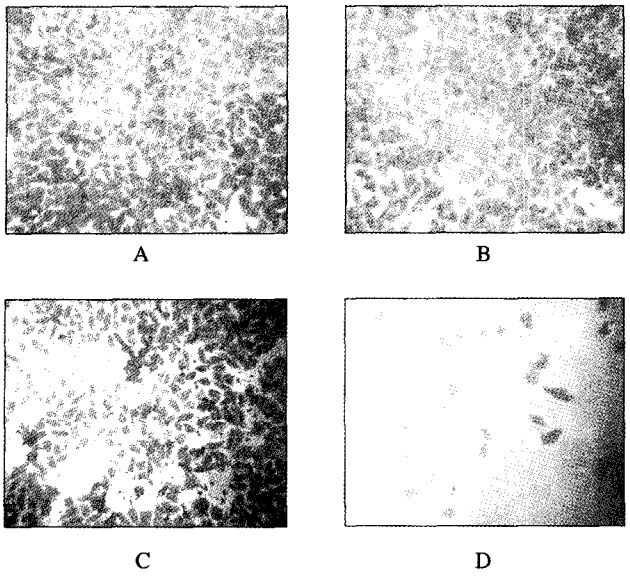

Fig. 9. Synergistic cytotoxicity *in vitro* due to the combination treatment with TRAIL and PFF ethanol extracts. A: A549 cells were treated with TRAIL alone (200 ng/mL) B: A549 cells were treated either with TRAIL (200 ng/mL) plus PFF ethanol extracts (5 μg/mL) C: A549 cells were treated either with TRAIL (200 ng/mL) plus PFF ethanol extracts (10 μg /mL) D: A549 cells were treated either with TRAIL (200 ng/mL) plus PFF ethanol extracts (20 μg /mL).

TRAIL-induces apoptosis in A549 cells showed an induced synergistic effect on the TRAIL-induced apoptosis in A549 cells, which were strongly resistant to TRAIL. These results suggest that PFF ethanol extracts might contain effective substances for TRAIL-induced apoptosis. Thus, the cytotoxic substance contained in ethanol extracts of PFF should be characterized and purified for the development of antitumor agents.

In summary, inhibitory substances against the viability of cancer cells are contained in the fruiting bodies of *Pleurotus ferulae*. Although these substances were simply extracted by ethanol, their cytotoxic effect was the highest in the lung cancer cells, A549.

CONCLUSION

In order to find the antitumor effects of *Pleurotus ferulae*, hot water an ethanol cell extracts were prepared from the fruiting bodies (PFF) and mycelium (PFM). The antitumor activities of these extracts were examined with three human solid carcinomas, a lung carcinoma (A549) and two cervical carcinoma (SiHa and HeLa). The cytotoxicities of the PFF and PFM extracts were determined by MTT assays.

When SiHa cells were incubated for varying periods of time with PFF and PFM extracts, the cytotoxicity of the PFF ethanol extracts was higher than the other extracts. These results indicated that the extracts from fruiting body of *P. ferulae* have antitumor substances.

When A549, SiHa and HeLa cells were incubated with different concentrations of the PFF and PFM extracts, the PFF ethanol extracts showed strong cytotoxicity against the A549 cells at concentrations over 10 μg/mL and against the SiHa and HeLa cells at concentrations over 40 μg/mL. However, the differences in the cytotoxic effects of the hot water and ethanol extracts of PFM and the hot water extracts of PFF on all 3 cancer cells were not significant. Also, the PFF ethanol extracts induced a synergistic effect on the TRAIL-induced apoptosis in A549 cells, which were strongly resistant to TRAIL. These results indicated that the PFF ethanol extracts were the most prominent antitumor agents toward the lung cancer cells (A549).

Thus, a cytotoxic substance contained in the PFF ethanol extracts should be characterized and purified for the development of antitumor agents.

In summary, inhibitory substances against the viabilities of cancer cells are contained in the fruiting bodies of *Pleurotus ferulae*. Although these substances were simply extracted by ethanol, their cytotoxic effect was highest toward the lung cancer cells, A549.

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