

Inhibition of Growth and Induction of Apoptosis in Human Cancer Cell Lines by an Ethyl Acetate Fraction from Shiitake Mushrooms

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ABSTRACT

Objective: Shiitake (*Lentinus edodes*) mushrooms have been reported to have cancer-preventing properties. However, little research has been conducted verifying the antitumor activities of “mycochemicals” in shiitake mushrooms. In this study, potential roles of an ethyl acetate fraction from shiitake mushrooms were investigated by *in vitro* bioassays.

Design: The activities of an ethyl acetate fraction were evaluated by [3-(4,5-dimethylthiazol-yl)-2,5-diphenyl-tetrazolium bromide] (MTT), apoptosis bioassay, cell cycle analysis, and Western blot analysis using two human breast carcinoma cell lines (MDA-MB-453 and MCF-7), one human nonmalignant breast epithelial cell line (MCF-10F), and two myeloma cell lines (RPMI-8226 and IM-9).

Results: Concentration-dependent antiproliferative effects of the fraction were observed in all cell lines using the MTT assay. Approximately 50 mg/L concentration of the fraction induced apoptosis in 50% of the population of four human tumor cell lines and the fraction-induced apoptosis may have been mediated through the pro-apoptotic bax protein which was up-regulated. Cell cycle analysis revealed that the fraction induced cell cycle arrest by significant decrease of S phase, which was associated with the induction of cdk inhibitors (p21) and the suppression of cdk4 and cyclin D1 activity. Compared to malignant tumor cells, nonmalignant cells were less sensitive to the fraction for the suppression of cell growth and regulation of bax, p21, cyclin D1, and cdk4 expression. A 51% antiproliferative effect occurred at the highest concentration of the fraction (800 mg/L).

Conclusions: These data suggest that inhibition of growth in tumor cells by “mycochemicals” in shiitake mushrooms may result from induction of apoptosis.

INTRODUCTION

Shiitake mushrooms (*Lentinus edodes*) have been described as having antiviral, antibiotic, anti-inflammatory, antihypertensive, and anticancer properties,^{1–3} and have been popular as a health food for thousands of years in the East and more recently in the West.

Orally administered shiitake mushroom fruiting bodies have been reported to have anticarcinogenic effects against some tumor types and that these effects appear to be strain specific.^{4,5} Diets containing shiitake mushrooms significantly reduced the growth of Sarcoma-180 cells in female ICR mice and MM-46 carcinoma in C3H mice, but had little effect on growth of other tumor types such as B-16 melanoma, Lewis lung carcinoma,

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or meth-A fibrosarcoma.^{4,5} Moreover, the anti-cancer effects of the shiitake diets were found to be accompanied by activation of macrophages and increased cytotoxic effects of both NK and killer T cells,^{4,5} suggesting that one possible mechanism of this tumor-inhibiting activity may involve boosting immune surveillance.

With regard to identification of anticancer constituents in shiitake mushrooms, an antitumor polysaccharide was isolated from fruiting bodies and named lentinan.^{6,7} Its structure was confirmed as β -(1 \rightarrow 3)-glucans (C₆H₁₀O₅)_n, with the mean molecular weight of 5×10^5 kDa.⁸ Lentinan has been established to be a major antitumor-active component in shiitake mushrooms. However, mammals lack the enzyme, β -1 \rightarrow 3-glucanase, required to digest lentinan and purified lentinan has been reported to possess no antitumor activity when administered orally.^{3,9} One study has been conducted on fractionation of chemical constituents in shiitake mushrooms and orally administered the fractions.⁴ When tumor-bearing mice received the ethyl ether-ethanol fraction in the diet, the growth of tumors was inhibited by 24.7%. It appears that in addition to anticancer polysaccharides, shiitake mushroom also contains as yet uncharacterized ethyl ether-ethanol soluble anticancer mycochemicals. To date, very little research has been conducted on health effects of the ethyl ether-ethanol soluble portion from shiitake mushrooms.

In the present study, the anticancer effects of an ethyl acetate fraction from shiitake mushrooms were assessed against human breast carcinoma cell lines (MDA-MB-453 and MCF-7), the human nonmalignant breast epithelial cell line (MCF-10F), and myeloma cell lines (RPMI-8226 and IM-9). The effects on the suppression of cell growth were measured by [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. Apoptosis assay and cell cycle analysis were used to elucidate the basis of the antiproliferative properties of the fraction. In addition, the authors evaluated the effects of the fraction on the levels of Bax, p21, cyclin D1, and cdk4 in the treated cells by Western blot analysis.

MATERIALS AND METHODS

Preparation of an ethyl acetate fraction from shiitake mushrooms

Log-grown shiitake mushrooms were obtained from Shirley Community Development Corporation (Shirley, AR). Frozen shiitake mushrooms (2000 g) were extracted with 80% aqueous methanol (3000 mL) and 50% aqueous methanol (3000 mL) at 5°C. The extracts were combined and concentrated on a rotary evaporator under reduced pressure at room temperature until the methanol was removed. The aqueous extract (200 mL) was partitioned with ethyl acetate two times (300 mL \times 2). The combined ethyl acetate fraction was evaporated under reduced pressure at room tem-

perature followed by drying in a freeze-dryer. The ethyl acetate fraction represented 2.12% of frozen weight of the shiitake mushrooms.

Cell cultures and treatment

Two human breast carcinoma cell lines (ER-negative MDA-MB-453 and ER-positive MCF-7), one human nonmalignant breast epithelial cell line (ER-negative MCF-10F), and two myeloma cell lines (EBV-negative RPMI-8226 and EBV-positive IM-9) were obtained from the American Type Culture Collection (Rockville, MD). The cell lines were cultured as described in the instructions from the American Type Culture Collection. The fraction from shiitake mushrooms was dissolved in dimethylsulfoxide (DMSO) in different concentrations and mixed with medium before treatment. The final concentration of DMSO in treated cell medium was 0.1% (0.1% DMSO alone as control).

Cell proliferation assay

The effects of the fraction from shiitake mushrooms on cell proliferation were measured by MTT assay. The MTT assay of myeloma cell lines was conducted according to published procedure¹⁰ and the MTT assay of human breast carcinoma cell lines was carried out using the methods described previously.¹¹ The cells were treated with 0, 0 (with 0.1% DMSO), 12.5, 50, 200, and 800 mg/L fraction and incubated for 24, 48, or 72 h before treatment with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma, St. Louis, MO)].

Apoptosis assays

The apoptotic effect of the fraction from shiitake mushrooms was determined by staining cells with Annexin V-fluorescein isothiocyanate (FITC) (BD Biosciences, Palo Alto, CA) and propidium iodide (PI) (Sigma).¹² The cells were cultured in medium containing various concentrations of the fraction for 24, 48, or 72 h before apoptosis assays. Data plots were generated from analysis of ungated data and representative dot plots indicate apoptotic tumor cells determined by PI and Annexin V-fluorescein isothiocyanate (FITC) staining. The total apoptotic cells are the sum of PI⁻/Annexin V⁺ (newly apoptotic) and PI⁺/Annexin V⁺ (already apoptotic) cell populations.¹²

Cell cycle analysis

To determine the effects of the fraction from shiitake mushrooms on cell cycle progression, 5-bromo-2'-deoxyuridine [BrdU] (Sigma) incorporation was used.¹³ The cells were cultured in medium containing various concentrations of the fraction for 24 h before cell cycle analysis. Cell cycle distribution was analyzed by flow cytometry.¹³

Protein extraction and Western blot analysis

Two human breast carcinoma cell lines (MDA-MB-453 and MCF-7) and one human nonmalignant breast epithelial cell line (MCF-10F) were used in Western blot analysis. The cells were incubated in 75-cm² flasks and treated with the fraction from shiitake mushrooms at 100, 400, or 800 mg/L concentrations for 24 h. Protein from treated cells were fractionated by electrophoresis on 15% SDS-PAGE and electrotransferred to immunoblot PVDF membranes (Bio-Rad laboratories, Hercules, CA). PVDF membranes incubated with appropriate dilutions of primary antibodies in blocking buffer: anti-cdk4 (1:600), anti-p21^{WAF1} (1:500), anti-Bax (1:600), and anti-cyclin D1 (1:2000). Subsequently, the membranes were incubated with the peroxidase-conjugated anti-rabbit secondary antibody diluted in blocking buffer: 1:6000 for anti-cdk4, 1:5000 for anti-p21^{WAF1}, and anti-Bax; or the peroxidase-conjugated anti-mouse secondary antibody 1:3000 for anti-cyclin D1. Antibody-bound proteins were visualized by ECL plus Western blotting analysis system (Amersham Pharmacia Biotech, UK). The primary antibodies against cdk4, p21^{WAF1}, Bax, and two secondary antibodies were purchased from Santa-Cruz Biotechnology (Santa Cruz, CA), and anti-cyclin D1 antibody was from Cell Signaling Technology (Beverly, MA).

LC-MS analysis

The fraction from shiitake mushrooms was directly analyzed by LC-MS. LC-MS was performed using a Bruker Esquire-LC multiple ion trap mass spectrometer equipped with an Agilent 1100 series liquid chromatograph. An HP ChemStation was used for data collection and manipulation. A 150 × 4.6 mm i.d. Eclipse XDB-C8 column (Agilent Technologies, Wilmington, DE) was used with LC solvent at a flow rate of 0.8 mL/min. The LC gradient was 0.1% formic acid/acetonitrile (solvent B) in 0.1% formic acid/H₂O (solvent A) as follows: 10% to 15% in 15 min; 15% to 18% from 15 to 16 min; 18% to 22% from 16 to 33 min; 22% to 40% from 33 to 40 min; held at 40% from 40 to 45 min; 40% to 42% from 45 to 49 min; 42% to 45% from 49 to 50 min; 45% to 50% from 50 to 65 min; 50% to 65% from 65 to 75 min; 65% to 85% from 75 to 76 min; 85% to 100% from 76 to 80 min; held at 100% from 80 to 85 min and finally back to 10% in 90 min. Mycochemicals in the eluant were analyzed by MS with positive ion modes. LC-MS analysis was carried out using the methods described previously.¹⁴

Statistical analysis

Results are means ± SD for at least three replicate determinations for each assay. Significant differences between two groups were determined by student's *t*-test. Differences with *p*-values <0.05 were considered significant.

RESULTS

Antiproliferative properties of the ethyl acetate fraction

The authors assessed antiproliferative effects of the fraction from shiitake mushrooms against five cell lines using the MTT assay. Following incubation with the fraction at 0 without DMSO, 0 with 0.1% DMSO, 12.5, 50, 200, and 800 mg/L concentrations for 24 h, the fraction led to a concentration-dependent reduction (*p* < 0.05) of relative cell viability (Fig. 1). IC₅₀s for the suppression of cell growth for two human breast carcinoma cell lines (MDA-MB-453 and MCF-7) and two myeloma cell lines (RPMI-8226 and IM-9) were 289 ± 108, 179 ± 34, 444 ± 40, and 477 ± 88 mg/L, respectively. However, at the highest concentration of the fraction (800 mg/L), only 51% antiproliferative effect occurred in the nonmalignant breast epithelial cell line MCF-10F.

Fraction-induced apoptosis

To evaluate the fraction-induced apoptosis, two human breast carcinoma cells and two multiple myeloma cells were treated with the ethyl acetate fraction from shiitake mushrooms at 0 without DMSO, and 0 with 0.1% DMSO, 12.5,

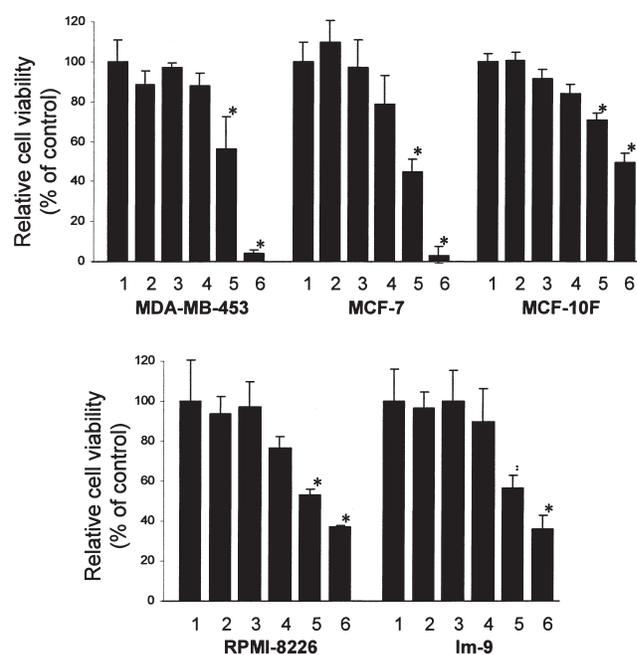


FIG. 1. Antiproliferative effects on 5 cell lines by four concentrations of an ethyl acetate extract from shiitake (*Lentinus edodes*) mushrooms for 24 hours of treatment as determined by [3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. 1 = Medium as control; 2 = 0.1% dimethylsulfoxide; 3 = 12.5 mg/L; 4 = 50 mg/L; 5 = 200 mg/L; 6 = 800 mg/L. Each value represents the mean ± standard deviation of more than three replicates. **P* = values <0.05, which were considered significant versus control.

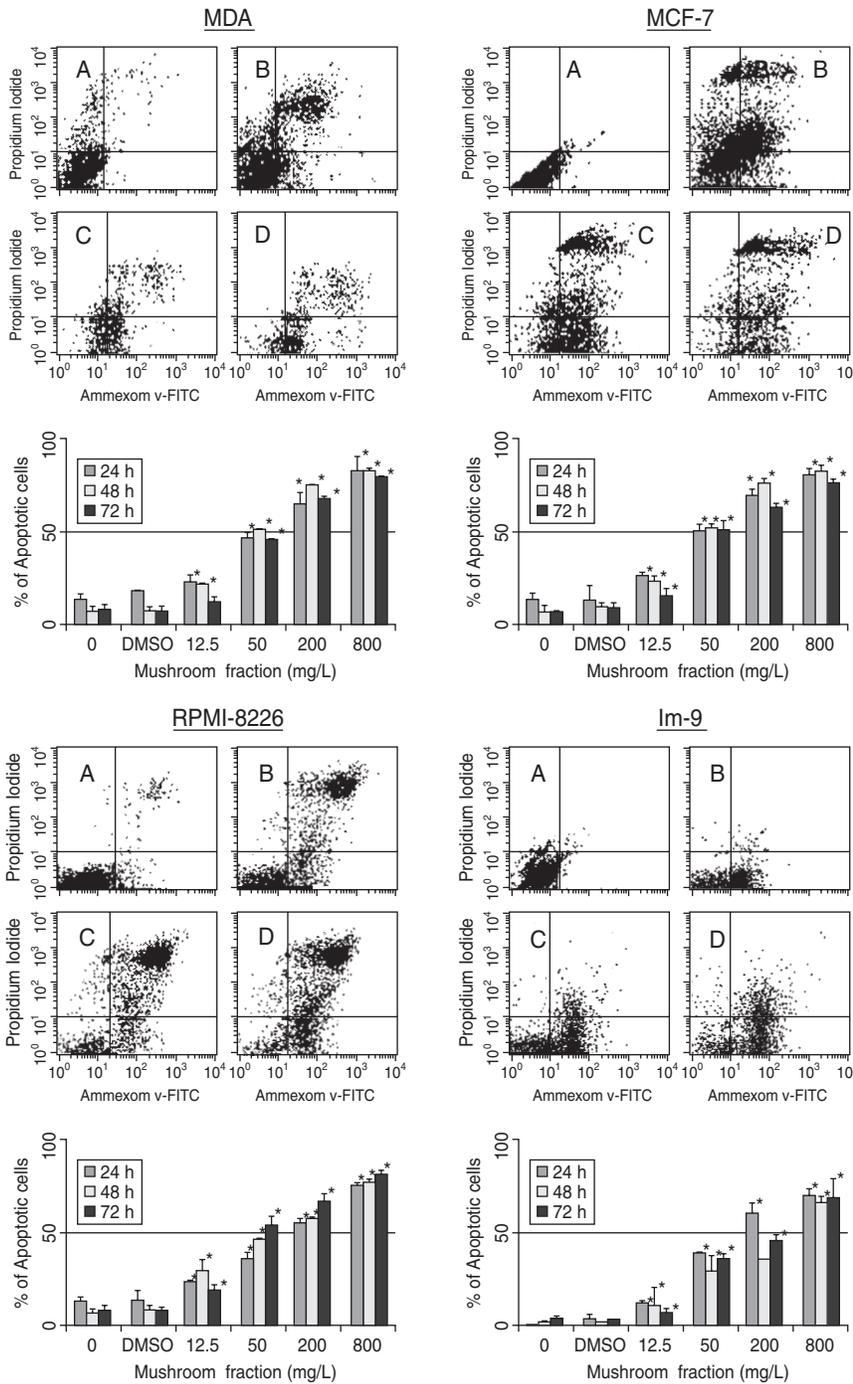


FIG. 2. Ethyl acetate extract from shiitake (*Lentinus edodes*) mushroom-induced apoptosis in two human breast carcinoma cell lines and two myeloma cell lines. Representative dot plots showing apoptotic tumor cells determined by PI and Annexin V-fluorescein isothiocyanate (FITC) staining. Live cells (PI⁻/Annexin V⁻), newly apoptotic cells (PI⁻/Annexin V⁺) and already apoptotic cells (PI⁺/Annexin V⁺) are located at LL, LR, and UR, respectively. In each part of this figure where letters appear: A = control; B = 50 mg/L for 48 hours; C = 200 mg/L for 48 hours; and D = 800 mg/L for 48 hours. The percentages of apoptotic cells are the sum of PI⁻/Annexin V⁺ (newly apoptotic cells) and PI⁺/Annexin V⁺ (already apoptotic) cells population by Flow cytometry analysis. Each value represents the mean ± standard deviation of more than three replicates. **P* values <0.05, which were considered significant versus control. DMSO, dimethylsulfoxide.

50, 200, and 800 mg/L concentrations. The cells were sequentially stained with annexin V-FITC and PI following 24, 48, and 72 h of fraction treatments. The pattern of the population stained with annexin V-FITC and/or PI of the treated cells and their controls were quite different, which indicated that the ethyl acetate fraction led to significant induction of apoptosis in human breast carcinoma cells and myeloma cells. The apoptotic effect of the fraction was dose-dependent. The degrees of apoptosis were not significantly

different between human breast carcinoma cells and myeloma cells. Figure 2 shows approximately 50% of cells bind annexin V-FITC and/or PI at 50 mg/L concentration of the ethyl acetate fraction from shiitake mushrooms.

Fraction arrests the cell cycle of tumor cells

The cell cycle distribution of two human breast carcinoma cell lines (MDA-MB-453 and MCF-7) and two myeloma

cell lines (RPMI-8226 and IM-9) were examined 24 h after treatment of the ethyl acetate fraction from shiitake mushrooms at 0 (0.1% DMSO), 50, 200, and 800 mg/L. The fraction-treated cells exhibited prominent increases in the percentage of cells arresting in G0/G1 of all four cells (Fig. 3). The effect of the fraction was dose-dependent.

Expression of *bax*, *cdk4*, *cyclin D1*, and *p21^{WAF1}*

To elucidate the mechanisms of the effects of the ethyl acetate fraction from shiitake mushroom on apoptosis and cell cycle distribution of the tumor cells, MDA-MB-453, MCF-7, and MCF-10F cells were treated with the fraction at 0 (0.1% DMSO), 100, 400, and 800 mg/L concentrations and the expression of related proteins in the treated cells were examined by Western blot analysis. As shown in Figure 4, the levels of *bax* and *p21* protein expression were increased and, in contrast, the levels of *cyclin D1* and *cdk4* expression were decreased 24 h after fraction treatment. As expected, nonmalignant breast ep-

ithelial cells MCF-10F were least sensitive to mushroom extract.

LC-MS/MS fingerprint of the ethyl acetate fraction from mushrooms

The ethyl acetate fraction was analyzed by LC-MS and total ion chromatogram (TIC) from positive MS was established as the fingerprint of the fraction. Although the identification of the chemical structures of bioactive compounds has not been accomplished as yet, the fingerprint indicated numerous components (peaks overlapped in TIC chromatogram) in the fraction (Fig. 5). The base peaks in the mass spectra of TIC peaks **a** through **j** were shown in Figure 5 for the characterization of the fingerprint. Preparation of fraction was repeated several times and the variability of LC-MS profiles of different fractions from same preparation procedure did not have significant difference on the basis of the comparison of the number and areas of peaks in LC-MS profiles.

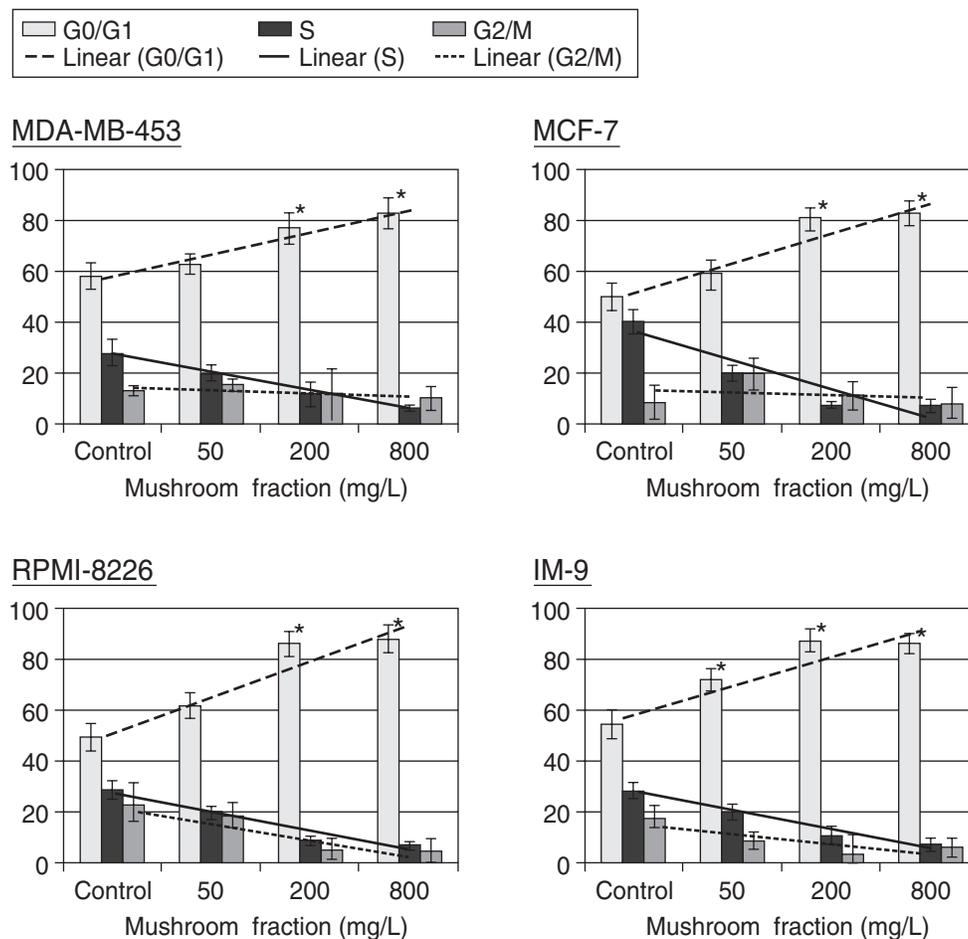


FIG. 3. Cell cycle analysis of two human breast carcinoma cell lines and two myeloma cell lines after exposure to an ethyl acetate extract from shiitake (*Lentinus edodes*) mushrooms for 24 hours. Cell cycle distribution was analyzed by flow cytometry. **P* values < 0.05 were considered significant versus control.

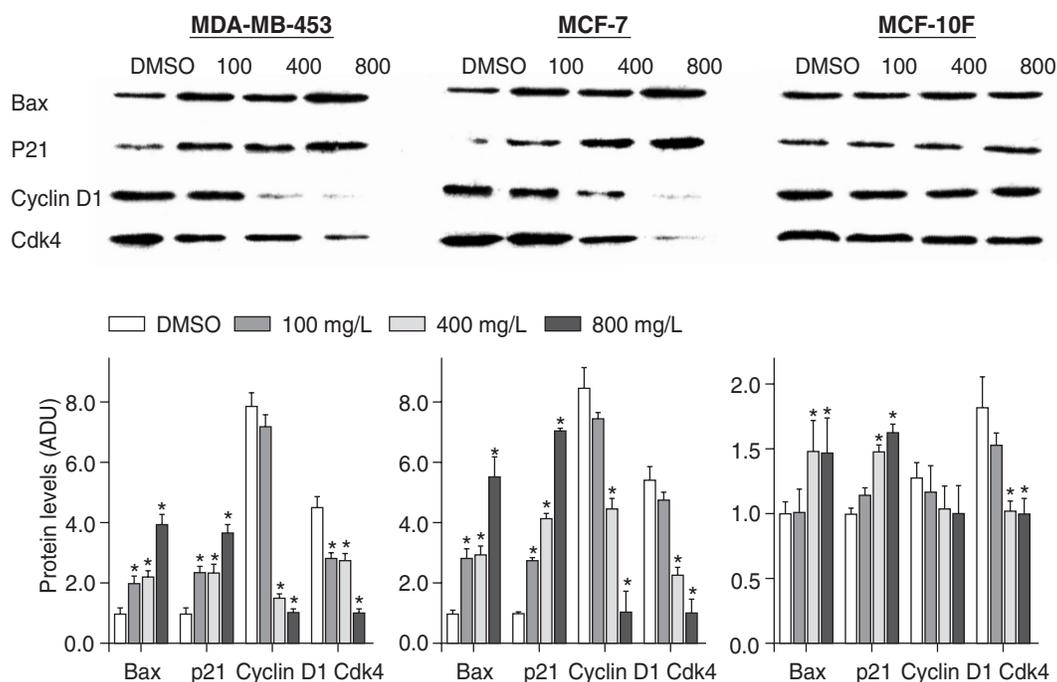


FIG. 4. Expression of bax, p21, cyclinD1, and cdk4 in MDA-MB-453, MCF-7, and MCF-10F cells with Western blot analysis. The cells were treated with the ethyl acetate fraction from shiitake (*Lentinus edodes*) mushrooms in various doses for 24 hour. Each value represents the mean \pm standard deviation of three replicates. **P* values <0.05, which was considered significant versus control.

DISCUSSION

Shiitake mushrooms are a good source of protein, fiber, and certain vitamins (thiamine, riboflavin, niacin, D₂, B₂, and B₁₂). Improved growing techniques have resulted in increased marketing of low-priced, high-quality shiitake mushrooms and the consumption by Americans is at an all-time high.

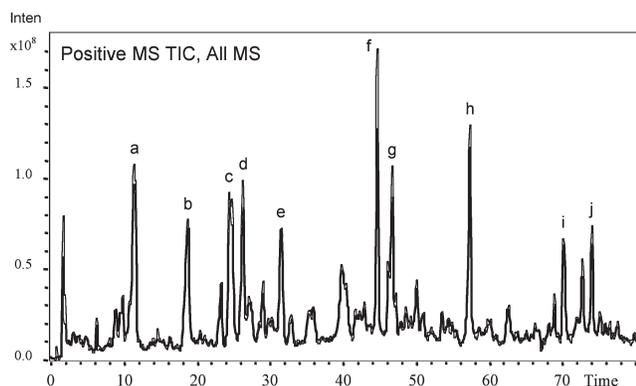


FIG. 5. LC-MS fingerprint of the ethyl acetate fraction from shiitake (*Lentinus edodes*) mushrooms that have anticancer activities. The base peaks in the mass spectra of peaks **a** through **j** were at *m/z* 277, 279, 498, 498, 377, 377, 413, 379, 737, and 313, respectively, and used for the characterization of the fingerprint. Inten, intensity.

Shiitake mushrooms have been reported to have cancer-preventing properties.^{4,5,15} Most cancer-related research on shiitake mushrooms has been conducted on the β -(1 \rightarrow 3)-glucans polysaccharides (named lentinan), which are promoted as major anticancer components in the shiitake mushroom and are being used in some parts of the world as agents to improve the outcome of cancer therapy. However, mammals lack the necessary enzyme, β -1 \rightarrow 3-glucanase, and lentinan has been reported to possess no antitumor activity when administered orally.^{3,9} These data suggest that mycochemicals may be responsible for the anticancer effects of the shiitake mushroom. This report describes the antitumor activities of mycochemicals in the ethyl acetate fraction (nonpolysaccharide) from shiitake mushrooms by *in vitro* bioassay in two human breast carcinoma cell lines (MDA-MB-453 and MCF-7), one nonmalignant breast epithelial cell line (MCF-10F), and two myeloma cell lines (RPMI-8226 and IM-9).

Antiproliferative properties of the ethyl acetate fraction from shiitake mushrooms were observed in all cell lines using the MTT assay. Antiproliferative effects of the fraction were concentration-dependent and the degree of antiproliferative activity in human breast carcinoma cell lines was higher than in nonmalignant breast epithelial cell line (Fig. 1). Similarly, there was a dose-dependent increase of apoptosis in the treated cells (Fig. 2), consistent with the cell growth suppression detected in the MTT assay. Fraction-induced apoptosis occurred at lower concentrations than the

concentration for the antiproliferative effect determined by MTT because MTT was used to determine the dead cells and the apoptosis bioassay measured the damaged cells and dead cells together. Western blot analysis showed that treatment with the fraction from shiitake mushrooms enhanced expression of the proapoptotic bax protein levels in human breast carcinoma cell lines (Fig. 4), suggesting that fraction-induced apoptosis of the cells might be mediated by increasing bax expression.

Cell cycle analysis showed an increased proportion of cells in G0/G1 phase, indicating an arrest of cell cycle progression from the G1 to the S phase (Fig. 3). Cell cycle progression is accelerated by cyclins and cdks, and decelerated by cdk inhibitors, such as p21.¹⁶ The D-type cyclins (cyclin D1, D2, and D3) are involved in regulation of transition from G1 to S during cell cycle. In this study, MDA-MB-453 and MCF-7 cells treated with the fraction showed upregulation of p21, and downregulation of cyclin D1 and cdk4. These results suggest that the fraction from shiitake mushrooms induces G1 arrest in cells via the induction of cdk inhibitors (p21), with the suppression of cyclin D1 and cdk4 activities. Compared to malignant cell lines, nonmalignant MCF-10F cells were less sensitive to effects of the fraction on the suppression of cell growth and the regulation of expression of cdk4, cyclin D1, and p21 (Fig. 4).

Concentrations of an ethyl acetate fraction from shiitake mushrooms that elicited bioactivity in this study were high (50 mg/L). However, LC-MS fingerprint revealed that this bioactive fraction consists of more than 100 mycochemicals (peaks) without major components (Fig. 5). It is reasonable to assume that the bioactivity in the fraction results from one or more specific mycochemicals, but not all mycochemicals in the fraction. The potency of the bioactive mycochemicals, alone or together, should be much higher than the potency of the whole fraction.

CONCLUSIONS

Shiitake mushrooms have been reported to have cancer-preventing properties and lentinan (β -1 \rightarrow 3-glucans polysaccharides) isolated from shiitake mushrooms are being used in some parts of the world as an adjuvant in cancer chemotherapy. However, mammals lack the necessary enzyme, β -1 \rightarrow 3-glucanase, and lentinan has been reported to possess no antitumor activity when administered orally.^{3,9} The inhibitory activities of the ethyl acetate fraction from shiitake mushrooms against several human cancer cell lines were detected by *in vitro* bioassay in this study, which suggests that mycochemicals may be responsible for the anticancer effects of the shiitake mushroom in humans,¹⁻³ and in *in vivo* study.^{4,5} LC-MS analysis of the fraction reveals more than 100 chemicals in the fraction without quantitatively dominant mycochemical constituent. Clearly, further

work is needed to identify the antitumor mycochemicals in the ethyl acetate fraction from shiitake mushrooms and delineate their potency and mechanisms of action.

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