SPANDIDOS PUBLICATIONS

In vitro effects on proliferation, apoptosis and colony inhibition in ER-dependent and ER-independent human breast cancer cells by selected mushroom species

YU-HUAN GU and JESSICA LEONARD

Bastyr University, 14500 Juanita Drive NE, Kenmore, WA 98028, USA

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Abstract. Breast cancer is the most commonly diagnosed cancer among women in Western countries. Currently, there is no effective therapy for malignant estrogen-independent breast cancer. We have screened 38 species of edible mushroom on human estrogen-receptor positive (MCF-7) and estrogen-receptor negative (MDA-MB-231, BT-20) breast cancer cells to select potential agents with broad-spectrum antitumor activity against breast cancer cells. Water-based extracts of three mushroom species, *Coprinellus sp., Coprinus comatus, Flammulina velutipes* (CME, CCE and FVE respectively), were identified as novel anti-breast cancer agents. The anti-tumor activities include: 1) marked growth inhibition of both ER^+ and ER^- breast cancer cells; 2) induction of rapid apoptosis on both ER^+ and ER^- cells; 3) significant inhibition of MCF-7 tumor colony formation *in vitro*. The antiproliferative and cytotoxic activities of the three mushroom extracts were dose-dependent, regardless of the hormone receptor status of the cancer cells. The degree of produced cytotoxicity on ER^- breast cancer cells was very high, while the IC_{50} of mushroom extract CME was found to be as low as 40 μg/ml on MDA-MB-231 cells and the IC_{50} of mushroom extract FVE was only 30 μg/ml on BT-20 cells. More interestingly, mushroom extracts CME and FVE induced an exceptionally rapid apoptosis on MCF-7 and MDA-MB-231 detected by Annexin V-FITC within 2 h of treatment and DNA fragment end-labeling assay (TUNEL) in 5 h of treatment. Anchorage-independent growth assays indicated that the MCF-7 tumor colony formation rate was reduced by 60% in CCE- and CME-treated cells and nearly completely inhibited (99%) by FVE treatment. These results suggest that mushroom species *Coprinus comatus, Coprinellus sp*. and *Flammulina velutipes* contain potent antitumor compounds for breast cancer. Our finding is important due to the lack of chemotherapeutic and chemopreventive agents for ER^- human breast cancer.

Correspondence to: Dr Yu-Huan Gu, School of Natural Health Sciences, Bastyr University, 14500 Juanita Drive NE, Kenmore, WA 98021, USA
E-mail: yuhuang@bastyr.edu

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Introduction

Mushrooms have historically been used to maintain general human health worldwide. Since Ikekawa's group first noted that a water extract of shiitake fruiting bodies could inhibit transplanted tumors in mice in 1969 (1), some well-known mushroom species or the constituents derived from those mushroom have been tested against several major types of cancer, including stomach, lung, colon and liver (2,3). In addition to their excellent nutritional value, edible mushrooms represent an untapped resource for novel antitumor compounds. However, only a small percentage (<10%) of edible mushrooms have been scientifically evaluated for their antitumor properties (4), and little attention has been given to incorporating medicinal mushrooms into breast cancer treatment. In recent years, the use of mushroom-derived polysaccharides alone or as part of conventional chemotherapy regimens has achieved successful outcomes in patients with advanced cancer (5). An interest in using mushroom polysaccharides as a complementary approach to cancer treatment remains strong in complementary and alternative medicine (CAM) practice. In contrast to the immune modulating properties of polysaccharides, several studies have found that mushroom extracts contain other compounds (non-polysaccharides) which are directly cytotoxic to tumor cells (6-8). Our previous studies have also indicated that mushroom constituents other than polysaccharides in shiitake and oyster mushrooms have biological activity against murine skin carcinoma or human prostate carcinoma cells (9; unpublished data). These results suggest that mushrooms contain different bioactive compounds with a diverse biological activity, and scientific investigation is justified in evaluating the anticancer activity of edible mushrooms as novel chemopreventive and chemotherapeutic agents.

Breast cancer is the most commonly diagnosed cancer among women in Western countries. Comparatively little published literature addresses the activity of mushroom-derived compounds for breast cancer treatment or prevention. This may be related to the historically low incidence of breast cancer

in Asia, where most of the traditional medicinal mushroom species originate. Extracts of *Ganoderma lucidum* have been reported to suppress the motility of breast cancer cells (10). Extracts of *Agaricus blazei*, related to the white button mushroom, inhibit breast cancer cell proliferation via suppression of aromatase activity (11,12). In addition to these *in vitro* experiments, there has been some attempt to evaluate mushroom polysaccharides in breast cancer treatment. A polysaccharide known as PSK was reported to extend the survival of Japanese patients with estrogen-receptor-negative (ER^-), stage IIA T2N1 adenocarcinomas, but not estrogen-receptor-positive (ER^+) breast tumors (13). This finding was not corroborated by Morimoto *et al*, in a larger clinical trial (14).

We have screened thirty-eight species of edible mushroom (cultivated or non-cultivated) for their potential as anti-breast cancer candidates. Our preliminary studies have identified three mushroom species (*Coprinellus sp., Coprinus comatus* and *Flammulina velutipes*) with potent antiproliferative and cytotoxic activities in both ER^+ (MCF-7) and ER^- (BT-20, MDA-MB-231) human breast cancer cell lines. Since there have been no previous reports of the anti-tumor activities of these mushroom species, and the degree of anticancer activity has not been seen with other mushroom extracts in breast cancer cell lines, our study aims to evaluate the effects of these selected mushroom species on proliferation, apoptosis and tumor colony inhibition in human ER-positive and -negative breast cancer cells.

Materials and methods

Mushroom extracts and cell lines. Thirty-eight species of fresh mushrooms were either collected in the greater Seattle area or were obtained from supermarkets for those species that are commercially cultivated. Fresh mushrooms were homogenized with sterile distilled water (1:5 w/v) in a blender and the samples were incubated in a water bath at a series of temperatures (20˚C, 40˚C and 80˚C) for 2 h. Aqueous mushroom extracts were obtained after centrifugation of homogenized mushroom samples at 1700 x g and sterilization of the extract through a 0.2-μm sterile filter. The extracts were stored at -70˚C. Dried samples of the mushroom extract were obtained by lyophilizing the aqueous mushroom extracts.

Human breast cancer cell lines, MDA-MB-231 (ER^-) and MCF-7 (ER^+), were obtained from American Type Culture Collection (Manassas, VA). Another ER^- cell line, BT-20, was a generous gift from Dr Daesong Yim (Bastyr University). Cells were cultured in Minimum Essential Medium supplemented with 10% fetal bovine serum at 37˚C in a humidified atmosphere containing 5% CO_2.

MTS assay. The cytotoxicity induced by each mushroom extract was examined by using CellTiter 96® Aqueous Cell Proliferation Assay (Promega, Madison, WI). Tumor cells were seeded onto the wells of a 96-well cell culture plate (5000 cells/100 μl/well) and the plate was incubated for 24 h (37˚C, 5% CO_2). Specific mushroom extracts were premixed with fresh culture medium at different concentrations prior to introduction into the cell culture. Mixed medium (100 μl) was added to each well, bringing the total volume of liquid to 200 μl per well. Each dose of mushroom extract was tested for cytotoxic effect on BT-20, MCF-7 and MDA-MB-231 cells in triplicate and experiments were repeated at least three times. The cells were incubated at 37˚C, 5% CO_2 for 24 h prior to cell growth assessment. Cell viability was determined using the MTS dye uptake assay by incubating the cells with 20 μl of CellTiter 96 cell proliferation assay solution for 2 h (37˚C). The cell culture wells without added mushroom extract served as an untreated control and the cell culture wells with the antitumor drug, 5-fluorouracil (1 mM), served as a positive cytotoxic control. Cell viability was expressed as the ratio of the mean absorbance of treated cells to that of control cells. The sensitivity of tumor cells to the extracts (CCE, CME and FVE) were represented as IC_{50} values.

Annexin V-FITC apoptosis assay. Since phosphatidyl-serine (PS) externalization of the plasma membrane occurs in the early phases of apoptotic cell death, the high affinity PS binding protein Annexin V (conjugated to fluorescein isothiocyanate FITC) can be used to identify the early event of cell death mediated by the mushroom extracts (15). Counterstaining with propidium iodide (PI) allows the differentiation of necrotic and apoptotic cells. In this study, apoptotic and necrotic cells resulting from treatment with the mushroom extracts were quantified by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (Immunotech, France). In brief, MCF-7 and MDA-MB-231 cells were treated with the extracts CME and FVE which were prepared from the mushrooms *Coprinellus sp*. and *Flammulina velutipes* at 20˚C. The cells were harvested and washed with cold PBS by centrifuging at 1000 x g for 5 min at 4˚C. Cells ($2x10^5$) were resuspended in 100 μl of 1X binding buffer and stained with Annexin V-FITC (1 μl) and PI (5 μl) in the dark for 10 min at room temperature. Another 400 μl binding buffer was added to each tube at the end of staining. Cells without exposure to the mushroom extracts served as the untreated control and the cells treated with an anticancer drug, mitomycin C (MMC, 25 μM), were used as a positive control of induced apoptosis in MCF-7 and MDA-MB-231 cells. The samples were analyzed by a Coulter EPICS ELITE ESP flow cytometer (Coulter Co., Miami, FL).

TUNEL apoptosis analysis. TUNEL for assessment of apoptotic cell death was performed as described by Gavrieli's group (16). Nuclear DNA fragmentation induced by the mushroom extracts was measured using the DeadEnd Colorimetric TUNEL system (Promega). In brief, following exposure to CME or FVE at a series of concentrations for 5 h, MCF-7 and MDA-MB-231 cells were harvested by centrifuging at 1000 x g for 5 min at 4˚C. Glass slides containing a thin layer of the cells were fixed with 10% formine, incubated with TdT, visualized with nitroblue tetrazolium (Promega), and photographed. Tumor cells without exposure to the mushroom extracts were performed as untreated controls for both MCF-7 and MDA-MB-231.

Tumor colony inhibition assay. Colony formation in soft agar is a sensitive test of cell viability and proliferative potential. A modification of the two-layer soft agar culture system (17) was used to determine the effects of the mushroom extracts on inhibition of tumor colony growth of MCF-7 cells. The

The effect of mushroom extracts (CME, FVE, CCE) on inhibition of human breast cancer cell lines.

Mushroom extract or anticancer drug	IC_{50} (μg/ml) on breast cancer cell lines		
	BT-20	MCF-7	MDA-MB-231
CME	150	120	40
FVE	30	150	75
CCE	N[a]	450	400
5-FU[b]	2 mM	1 mM	N

[a]N, no IC_{50} value was found at a concentration of ≤2 mM. [b]5-FU, 5-fluorouracil, an anticancer drug.

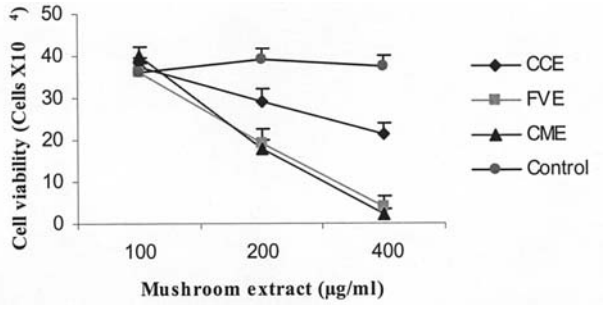

Figure 1. Dose responses of antitumor mushrooms on human breast cancer MCF-7 cells. Cells were cultured in minimal essential medium supplemented with 10% fetal bovine serum and exposed to aqueous extracts of *C. comatus* (CCE), *Coprinellus sp.* (CME) and *F. velutipes* (FVE) at a concentration of 100, 200 or 400 μg/ml for 24 h. Three replicates were used in each experiment and two separate tests were performed. Values shown are mean ± SD (n=6).

assay was performed in 60-mm Petri dishes with a base layer containing 0.6% agar in Minimum Essential Medium supplemented with 10% fetal bovine serum and 1 mM glutamine. This layer was overlaid with a second layer of 2 ml 0.2% agar (containing 20% fetal bovine serum, and 2 mM glutamine) mixed with 2 ml of a suspension of 3000 MCF-7 cells, with or without addition of 50 μg/ml mushroom extract (CCE, CME or FVE). The plates were incubated at 37˚C for 14 days, and stained with 0.5 mg/ml of p-iodonitrotrazolium violet (INT). The number of tumor colonies was counted using a microscope (x40) and the tumor colony forming efficiency (CFE, %) was calculated by comparison with the colony number of untreated control (as 100%).

Statistical analysis. In MTS and tumor colony inhibition assay, the result of each treatment is the mean ± standard deviation (SD) from 3 independent wells or plates. Cell viability data is representative of three individual experiments with similar results.

Results

Effects of mushroom extracts on growth inhibition of BT-20, MCF-7 and MDA-MB-231 breast cancer cells. A total of 38 aqueous extracts prepared from fresh mushroom fruiting bodies were screened for their potential activity against human breast cancer cell lines (ER^+: MCF-7, ER^-: BT-20 and MDA-MB-231). Three mushroom extracts (CCE, CME and FVE), prepared at 20˚C from the mushrooms, *Coprinus comatus, Coprinellus sp*. and *Flammulina velutipes* respectively, showed significant growth inhibition on all breast cancer cell lines, regardless of the hormone receptor status of the cancer cells at 24 h (Table I). Aqueous *F. velutipes* extract (FVE) exhibited greater inhibition of the ER^- breast cancer cell lines, BT-20 and MDA-MB-231, than in the ER^+ MCF-7 cells. The 50% growth inhibition concentration (IC_{50}) of FVE was as low as 30 μg/ml on BT-20 cells. The extract of *Coprinellus sp*. (CME) did not show ER-specific preference in the growth inhibition assay. Aqueous extract CCE from mushroom *C. comatus* displayed the least inhibition on breast cancer cell lines among the three extracts. The cytotoxic effects of these mushrooms on MCF-7 cells were demonstrated in a dose-responsive manner (Fig. 1). A similar dose-dependent action of the mushroom extracts was also observed in two other breast cancer cell lines, BT-20 and MDA-MB-231 (data not shown). Complete cell elimination resulted when MCF-7 cells were incubated with 400 μg/ml of CME or the same concentration of FVE for 24 h (Fig. 1). To verify that the anticancer activity observed in these mushrooms is not specimen-specific, we tested three batches of each mushroom species and no significant variation was found regarding the anti-breast cancer activity (data not shown).

Detection of apoptosis on MCF-7 and MDA-MB-231 by Annexin V-FITC/PI. Induced apoptosis was detected at as early as 2 h in ER^+ MCF-7 (Fig. 2) and ER^- MDA-MB-231 cells (Fig. 3) incubated with CME or FVE by Annexin V-FITC staining. Propidium iodide (PI) staining was used in conjunction with Annexin V-FITC for the detection of necrotic cells. Annexin V-negative/PI-negative, Annexin V-positive/PI-negative, Annexin V-positive/PI-positive, Annexin V-negative/PI-positive cells represent the viable cells, the cells in early apoptosis, late apoptosis, and necrosis, respectively. In MCF-7 cells, CME induced early stage apoptosis in 72% of the cell population and induced late stage apoptosis in another 11% of the cells at a dose of 112 μg/ml for 2 h. When 2-fold CME (225 μg/ml) was applied, the early-stage apoptotic cell population was less detected (61%) and the late-stage apoptotic cells increased to 32%, compared to that of low-dose CME treatment (Fig. 2c and d). A similar phenomenon was also observed with the FVE treatments (Fig. 2e and f). The MCF-7 cells that were not exposed to the extracts were analyzed as a negative control and cells treated with the anticancer drug, mitomycin C (MMC, 25 μM), were used as a positive control for apoptosis (Fig. 2a and b). Both CME and FVE significantly increased the apoptosis of MDA-MB-231 cells at all concentrations tested compared to the untreated cells or the cells treated with mitomycin C (25 μM) (Fig. 3). The results are consistent with those of cell viability assays as in Fig. 1. The results strongly demonstrate that CME and FVE induce apoptosis on MCF-7 and MDA-MB-231 cell lines in a rapid manner and may share a similar mechanism.

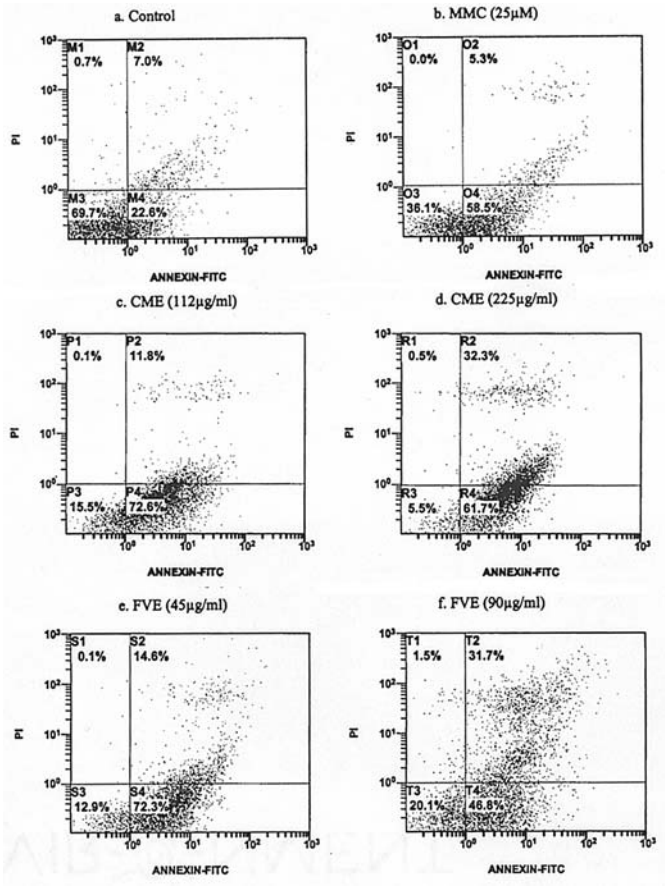

Figure 2. Detection of early stage of apoptosis induced by CME and FVE in ER+ breast cancer MCF-7 cells by Annexin V-FITC staining. The cells were stained with annexin V-FITC/PI and analyzed by flow cytometry following exposure to CME (112 μg/ml and 225 μg/ml) or FVE (45 μg/ml and 90 μg/ml) for 2 h. Anticancer drug, mitomycin C (25 μM), was used as a positive control for apoptosis of MCF-7 cells. Cells that were not treated with the mushroom extracts served as a negative control in the experiment. Results are representative of three individual experiments.

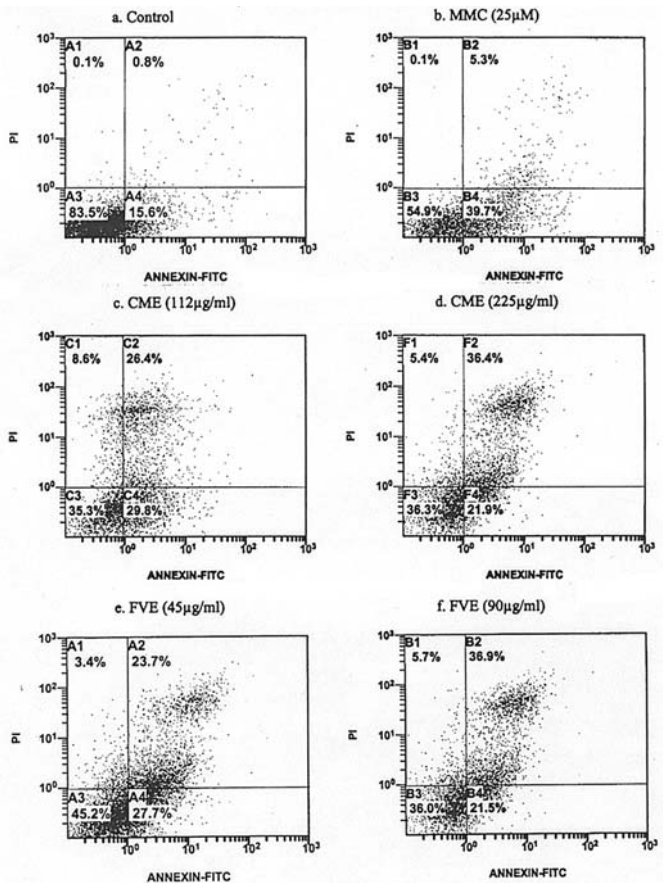

Figure 3. Detection of early stage of apoptosis induced by CME and FVE in ER- breast cancer MDA-MB-231 cells by Annexin V-FITC staining. The cells were stained with annexin V-FITC/PI and analyzed by flow cytometry following exposure to CME (112 μg/ml and 225 μg/ml) or FVE (45 μg/ml and 90 μg/ml) for 2 h. Anticancer drug, mitomycin C (25 μM), was used as a positive control for apoptosis of MDA-MB-231 cells. Cells that were not treated with the mushroom extracts served as a negative control in the experiment. Results are a representative of four individual experiments.

Detection of apoptosis on MCF-7 and MDA-MB-231 by TUNEL. Apoptosis triggered by CME and FVE in MCF-7 cells was confirmed by the TUNEL method. Cells were exposed to the extracts (400 μg/ml) for 5 h and analyzed by TdT-mediated X-dUTP nick end labeling. Colorimetric TUNEL stained apoptotic nuclei and fragmented DNA dark brown in the cells treated with CME and FVE (Fig. 4A), but did not stain untreated MCF-7 cells. The majority of the cell population was TUNEL-positive in CME-treated cells. The FVE-treated cells mainly lost integrity and appeared as stained debris at the same concentration (400 μg/ml). For ER- MDA-MB-231 cell lines, approximately 90% of cells were stained brown after 5 h of exposure to the mushroom extracts, CME or FVE, compared to the untreated cell control (Fig. 4B).

Inhibition of tumor colony formation by CME, CCE and FVE in vitro. Strong inhibition mediated by CME, CCE or FVE at 50 μg/ml was observed in MCF-7 colony formation and growth, compared to the untreated MCF-7 control (Fig. 5). The colony forming efficiency (CFE) was reduced to 40% and 41% in CME and CCE supplemented plates, respectively, compared to the untreated control (as 100% of CFE) (Table II). In FVE-supplemented soft agar medium, only 1% of colonies were able to grow *in vitro*, and the inhibition rate of colony formation was nearly 100% (Table II, Fig. 5). The mushroom extracts not only reduced cell colony numbers but dramatically decreased the tumor size of MCF-7 (Fig. 5). Interestingly, MCF-7 cells were more sensitive to the extracts in this anchorage-independent growth condition than the cells grown in an anchorage-dependent setting (Fig. 1, Table I).

Discussion

Although hormonal therapy is useful in preventing the development or recurrence of ER+ breast cancer, there are no effective therapies for malignant estrogen-independent breast cancer. Since approximately half of patients with advanced breast cancer ultimately progress to the estrogen resistant phenotype (18), and the mechanisms that enable breast cancer cells to acquire the estrogen-independent growth phenotype remain largely unknown, the most reliable remedies will have an ability to target the complex mix of cells in each individual cancer. CCE, CME and FVE, prepared from fresh fruiting bodies of the mushroom species *C. comatus, Coprinellus sp.* and *F. velutipes,* respectively, exhibited significant effects against human breast cancer cells, regardless of estrogen

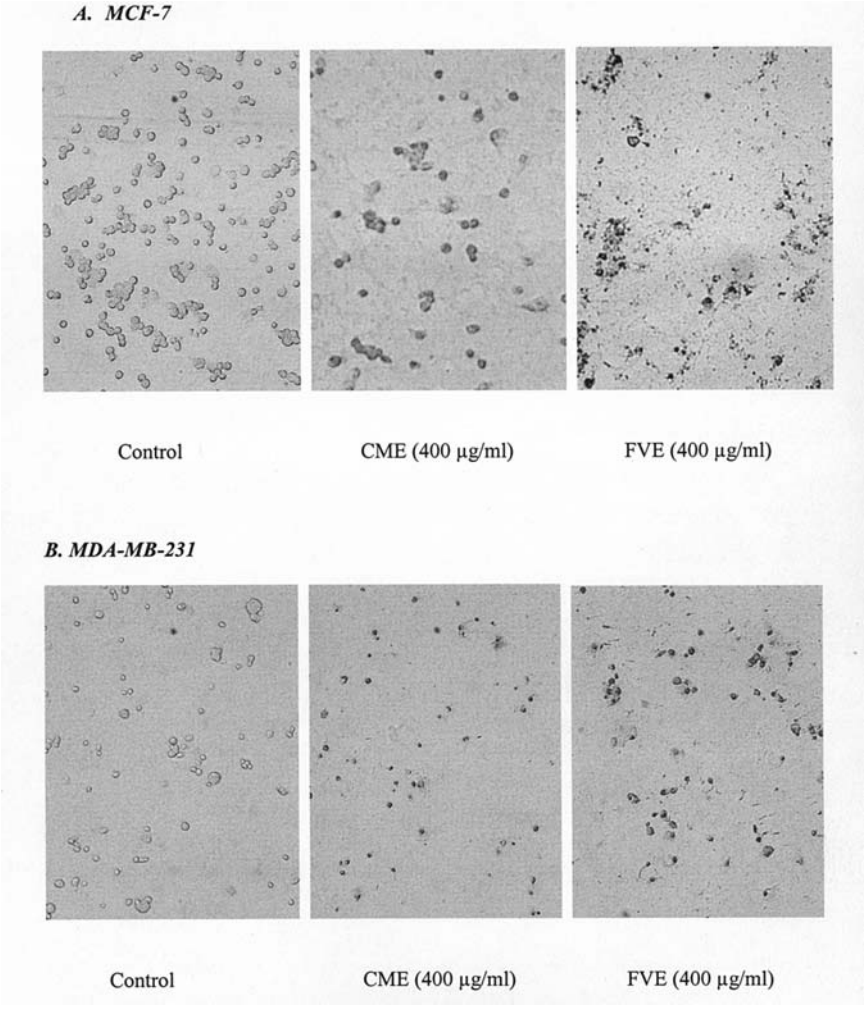

Figure 4. Detection of CME- and FVE-induced apoptosis on both ER^+ breast cancer MCF-7 cells (A) and ER^- breast cancer MDA-MB-231 cells (B) by DNA fragment TUNEL staining. Both cell lines were incubated with either CME or FVE (400 μg/ml) for 5 h and stained using a DeadEnd Colorimetric TUNEL system (Promega, Inc.). Apoptotic nuclei and fragmented DNA were stained dark brown in treated cells but not in untreated controls in MCF-7 (A) and MDA-MB-231 (B).

Table II. Inhibition of MCF-7 colony formation by mushroom extracts.

Mushroom extract (50 μg/ml)	Colony formation in soft agar medium	
	Colony ± SD/ Sq. inch[a]	CFE[b] (% of untreated control)
None	210±21	100
CME	85±7	40
FVE	2.5±2	1
CCE	103±11	41

[a]Colony numbers are means ± standard deviation of three replicate plates for each mushroom treatment. [b]CFE, colony forming efficiency.

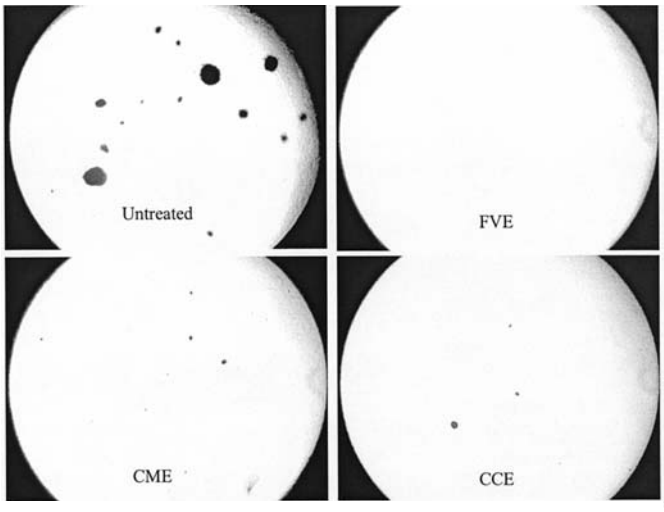

Figure 5. MCF-7 tumor colony inhibition mediated by CCE, CME and FVE *in vitro*. The cells were seeded into the soft agar medium supplemented with each extract at a concentration of 50 μg/ml. The assay was conducted in 60-mm Petri dishes with 3 dishes for each treatment. Cells that were plated without the addition of the mushroom extracts served as tumor colony growth controls. The dishes were incubated in a humidified incubator (37˚C) for two weeks and INT solution (1 ml per 60-mm dish) was added for additional 24 h. The tumor colony formation was observed under a microscope (x40) and photographed.

receptor status. Our study demonstrated a potential to explore novel anti-breast cancer agents that are multi-target driven and desperately needed to manage breast cancers that have achieved estrogen independence.

Medicinal mushroom species have been studied for their antitumor properties in several experimental animal models, and are used as a part of integrative/adjunctive cancer care in Asia. Detailed information on species with potential anticancer activity and the role of those mushrooms in tumor suppression are available in several recent reviews (2,19,20). *F. velutipes* is one of the most popular edible mushrooms in China and Japan where it is known as Enokitake. Considerable investigation has been given to the medicinal properties of this mushroom species previously, including its antitumor activity. Several polysaccharides were purified from the fruiting bodies of *F. velutipes* and displayed marked activity against solid tumors. EA6 (active compound: protein-bound polysaccharide), a hot water extract of *F. velutipes*, intensified host antitumor-immunity when combined with the murine leukemia L1210 vaccine in animal experiments (21). Other polysaccharides isolated from the mushrooms, such as EA3 and EA5, were also shown to possess antitumor activity in various experimental animal models (22,23). A glycoprotein, Proflamin, isolated from the culture mycelium of *F. velutipes* by ion exchange chromatography and molecular sieving, was markedly effective against the syngeneic tumors of B-16 melanoma and adeno-carcinoma 755 (24). However, Proflamin did not exhibit cytotoxic effects on tumor cells *in vitro*. Since the extract of *F. velutipes* that was used in this study, FVE, was prepared at a low temperature (20˚C) (in contrast to polysaccharide isolation, which requires boiling temperatures) and produced significant cytotoxicity on both ER^+ and ER^- tumor cells, we assume that the active compounds in FVE are not the well-known compounds identified from previous studies. Further investigation will be needed to isolate active compounds from FVE and characterize the chemical nature of these compounds. In contrast to the popularity of *F. velutipes*, the other two mushroom species, *C. comatus* and *Corinellus sp.*, were relatively unknown and seldom reported in published literature regarding their antitumor properties. The mushroom species in our study, containing potent anti-breast tumor activity *in vitro*, represent potential resources for exploring novel antitumor compounds for breast cancer.

Identifying natural compounds with the ability to induce apoptosis in cancer cells represents a large effort in the discovery of novel compounds for cancer chemoprevention and chemotherapy. Mycochemicals from mushroom origin, even though they are much less understood than phyto-chemicals from plant origin, have also shown potential in apoptosis induction in various tumor models. An acid-treated fraction (ATF) of *Agaricus blazei* extract directly inhibited tumor growth by inducing apoptotic processing in Balb/C mice (25). An ethanol extract of shiitake mushrooms selectively induced both apoptosis and G_1 arrest in murine skin carcinoma cells but not in skin normal cells (9). ß-glucan obtained from maitake mushroom induced >90% cytotoxic cell death in PC-3 cells at 480 μg/ml in 24-h treatment (26). However, this result was later contradicted by another research study which demonstrated that ß-glucan from maitake mush-rooms was only active for PC-3 cells in combination with carmustine (BCNU), an anticancer agent (27). A similar study by De Vere *et al* (28) also concluded that the polysaccharide/ oligosaccharide complex obtained from shitake mushroom extract was ineffective in the treatment of clinical prostate cancer when used alone. Interestingly, the Annexin V-FITC assay showed that CME and FVE induced a rapid apoptosis in MCF-7 and MDA-231 cells within 2 h. About 72% of MCF-7 cells were in the early stage of apoptosis and >10% of cells were in the late stage of apoptosis or necrosis (Fig. 2c) when CME (112 μg/ ml) was applied. Advanced apoptosis was observed in the same cell line when a high dose of CME (225 μg/ml) was added (Fig. 2d). Similar results were detected in ER^- MDA-MB-231 cells treated with the same concentrations of CME (Fig. 3c and d) or both cell lines with the addition of the mushroom extract, FVE. TUNEL staining also demonstrated that CME and FVE induced marked apoptotic events on MCF-7 and MDA-MB-231 cells within 5 h (Fig. 4). The rapid apoptosis induction observed in this study has rarely been reported in other studies involving induction of apoptosis by natural substances. Future analytical chemistry study of CME and FVE will probably identify a novel class of mushroom constituent with broad-spectrum antitumor activity for breast cancer chemoprevention or chemotherapy. Furthermore, a systematic approach to explore the mechanism of cytotoxicity, to identify the cellular and molecular targets associated with antitumor activity, and to assess the pharmacological value of mushroom extracts as chemotherapeutic agents for breast cancer is highly warranted.

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References

1. Ikekawa T, Uehara N, Maeda Y, Nankinishi M and Fukoka F: Antitumor activity of aqueous extracts of edible mushrooms. Cancer Res 29: 734-735, 1969.
2. Borchers AT, Stern JS and Hackman RM: Mushrooms, tumors, and immunity. Proc Soc Exp Biol Med 221: 281-293, 1999.
3. Ikekawa T: Beneficial effects of edible and medicinal mushrooms on health care. Int J Med Mushrooms 3: 291-298, 2001.
4. Hawksworth DL: Mushrooms: the extent of the unexplored potential. Int J Med Mushrooms 3: 333-340, 2001.
5. Kidd PM: The use of mushroom glucans and proteoglycans in cancer treatment. Altern Med Rev 5: 4-27, 2000.
6. Lam YW, Ng TB and Wang HX: Antiproliferative and anti-mitogenic activities in a peptide from puffball mushroom *Calvatia caelata*. Biochem Biophys Res Commun 289: 744-749, 2001.
7. Hsieh TC and Wu JM: Cell growth and gene modulatory activities of Yunzhi (Windsor Wunxi) from mushroom *Trametes versicolor* in androgen-dependent and androgen-insensitive human prostate cancer cells. Int J Oncol 18: 81-88, 2001.
8. Jiang J, Slivova V, Valachovicova T, Harvey K and Sliva D: *Ganoderma lucidum* inhibits proliferation and induces apoptosis in human prostate cancer cells PC-3. Int J Oncol 24: 1093-1099, 2004.
9. Gu YH and Belury M: Selective induction of apoptosis in murine skin carcinoma cells (CH72) by an ethanol extract of *Lentinula edodes*. Cancer Lett 220: 21-28, 2005.
10. Sliva D, Labarrere C, Slivova V, Sedlak M, Loyd FP and Ho NWY: *Ganoderma lucidum* suppresses motility of highly invasive breast and prostate cancer cells. Biochem Biophys Res Commun 298: 602-612, 2002.
11. Grube BJ, Eng ET, Kao YC, Kwon A and Chen S: White button mushroom phytochemicals inhibit aromatase activity and breast cancer cell proliferation. J Nutr 131: 3288-3293, 2001.
12. Chen S, Zhou D, Okubo T, Kao Y, Eng E, Grube B, Kwon A, Yang C and Yu B: Prevention and treatment of breast cancer by suppression aromatase activity and expression. Ann NY Acad Sci 963: 229-238, 2002.

, Hattori T and Akagi M: Randomized adjuvant trial to assess the addition of tamoxifan and PSK to chemotherapy in patients with primary breast cancer. Cancer 70: 2475-2483, 1992.
14. Morimoto T, Ogawa M and Orita K: Postoperative adjuvant randomized trial comparing chemo-endocrine therapy, chemotherapy and immunotherapy for patients with stage II breast cancer: 5-year results from the Nishinihon cooperative study group of adjuvant chemo-endocrine therapy for breast cancer of Japan. Eur J Cancer 32A: 235-242, 1996.
15. Vermes I, Haanen C, Steffens-Nakken H and Reutelingsperger C: A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. J Immunol Methods 184: 39-51, 1995.
16. Gavrieli Y, Sherman Y and Ben-Sasson SA: Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 119: 493-501, 1992.
17. Nomura Y, Tashiro H and Hisamatsu K: Differential effects of estrogen and antiestrogen on *in vitro* clonogenic growth of human breast cancers in soft agar. J Natl Cancer Inst 82: 1146-1149, 1990.
18. Dorssers LC, van der Flier S, Brinkman A, van Agthoven T, Veldscholte J, Berns EM, Klijn JG, Beex LV and Foekens JA: Tamoxifen resistance in breast cancer: elucidating mechanisms. Drugs 61: 1721-1733, 2001.
19. Wasser SP and Weis AL: Therapeutic effects of substances occurring in higher basidiomycetes mushrooms: a modern perspective (review). Crit Rev Immunol 19: 65-96, 1999.
20. Jong SC and Donovick R: Antitumor and antiviral substances from fungi. Adv Appl Microbiol 34: 183-261, 1989.
21. Otagiri K, Ohkuma T, Ikekawa T and Tanaka S: Intensification of antitumor-immunity by protein-bound polysaccharide, EA6, derived from *Flammulina velutipes* (Curt. ex Fr.) Sing. Combined with murine leukemia L1210 vaccine in animal experiments. J Pharmacobiodyn 6: 96-104, 1983.
22. Ikekawa T, Yoshioka Y, Emori M, Sano T and Fukuoka F: Studies on the antitumor activity of polysaccharides from *Flammulina velutipes* (Curt. ex Fr.) Sing. Cancer Chemother Rep 57: 85-86, 1973.
23. Ikekawa T, Ikeda Y, Yoshioka Y, Nakanishi K, Yokoyama E and Yamazaki E: Studies on antitumor polysaccharide, EA3 isolated from a Japanese edible mushroom, *Flammulina velutipes* (Curt. ex Fr.) Sing. II. The structure of EA3 and further purification of EA5. J Pharmacobiodyn 5: 576-581, 1982.
24. Ikekawa T, Maruyama H, Miyano T, Okura A, Sawasaki Y, Naito K, Kawamura K and Shiratori K: Proflamin, a new antitumor agent: preparation, physicochemical properties and antitumor activity. Jpn J Cancer Res 76: 142-148, 1985.
25. Fujimiya Y, Suzuki Y, Oshiman K, Kobori H, Moriguchi K, Nakashima H, Matumoto Y, Takahara S, Ebina T and Katakura R: Selective tumoricidal effect of soluble proteoglucan extracted from the basidiomycete, Agaricus blazei Murill, mediated via natural killer cell activation and apoptosis. Cancer Immunol Immunother 43: 147-159, 1998.
26. Fullerton SA, Samadi AA, Tortorelis DG, Choudhury MS, Mallouh C, Tazaki H and Konno S: Induction of apoptosis in human prostatic cancer cells with beta-glucan (Maitake mushroom polysaccharide). Mol Urol 4: 7-13, 2000.
27. Finkelstein MP, Aynehchi S, Samadi AA, Drinis S, Choudhury MS, Tazaki H and Konno S: Chemosensitization of carmustine with maitake beta-glucan on androgen-independent prostate cancer cells: involvement of glyoxalase I. J Altern Complem Med 8: 573-580, 2002.
28. De Vere WRW, Hackman RM, Soares SE, Beckett LA and Sun B: Effects of a mushroom mycelium extract on the treatment of prostate cancer. Urology 60: 640-644, 2002.