Original Research

Commonly consumed and specialty dietary mushrooms reduce cellular proliferation in MCF-7 human breast cancer cells

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Abstract

Worldwide, over one million women will be newly diagnosed with breast cancer in the next year. Moreover, breast cancer is the second leading cause of cancer death in the USA. An accumulating body of evidence suggests that consumption of dietary mushrooms can protect against breast cancer. In this study, we tested and compared the ability of five commonly consumed or specialty mushrooms to modulate cell number balance in the cancer process using MCF-7 human breast cancer cells. Hot water extracts (80°C for 2 h) of maitake (MT, *Grifola frondosa*), crimini (CRIM, *Agaricus bisporus*), portabella (PORT, *Agaricus bisporus*), oyster (OYS, *Pleurotus ostreatus*) and white button (WB, *Agaricus bisporus*) mushrooms or water alone (5% v/v) were incubated for 24 h with MCF-7 cells. Cellular proliferation determined by bromodeoxyuridine incorporation was significantly ($P < 0.05$) reduced up to 33% by all mushrooms, with MT and OYS being the most effective. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction, an often used mitochondrion-dependent marker of proliferation, was unchanged although decreased ($P > 0.05$) by 15% with OYS extract. Lactate dehydrogenase release, as a marker of necrosis, was significantly increased after incubation with MT but not with other test mushrooms. Furthermore, MT extract significantly increased apoptosis, or programmed cell death, as determined by terminal deoxynucleotidyl end labeling method, whereas other test mushrooms displayed trends of ~15%. The total numbers of cells per flask, determined by hemacytometry, were not different from control cultures. Overall, all test mushrooms significantly suppressed cellular proliferation, with MT further significantly inducing apoptosis and cytotoxicity in human breast cancer cells. This suggests that both common and specialty mushrooms may be chemoprotective against breast cancer.

Keywords: dietary mushrooms, breast cancer, cellular proliferation, apoptosis, MCF-7, *Agaricus bisporus*

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Introduction

Dietary mushrooms have been used globally for millennia to promote health and prevent and treat disease primarily via their multitude of medicinal qualities.[1] There are over 14,000 mushroom species but only ~3000 are edible, with ~700 exhibiting medicinal properties and <1% that are poisonous.[2,3] In general, medicinal dietary mushrooms have been shown to improve cardiovascular health, stimulate immune function, contribute to glucose homeostasis and to modulate detoxification, as well as exert antiallergic, antiviral, antibacterial, antifungal and anti-inflammatory activities.[4–6] Mushroom species or their constituent bioactive agents have been tested against several major forms of human cancer including stomach, colon, lung and liver and have been shown to be protective in numerous experimental models.[7] A particular research interest over the last several decades has been the ability of dietary mushrooms to reduce the occurrence of breast cancer. Despite this fact, less than 10% of edible mushrooms have been previously evaluated for antitumor properties in breast cancer treatment possibly due to the low incidence of breast cancer in Asia where the use of mushrooms as traditional medicine is prevalent.

Breast cancer is the most commonly diagnosed cancer, second only to skin cancer in women in the USA.[8] In 2009, there were ~192,000 new cases of breast cancer in women with ~40,000 deaths.[9] Accumulating evidence suggests that dietary mushrooms may decrease breast cancer incidence. In a case-control study of Chinese women with histologically confirmed breast cancer, dietary intake of 10 g of fresh mushrooms or 4 g of dried mushroom powder significantly decreased breast cancer in pre- and postmenopausal women.[10] In another study, 69% of breast cancer patients consuming whole maitake (MT) mushroom powder showed significant cancer regression and marked symptom improvement.[11] In cancer patients, MT D-fraction, an antitumor polysaccharide fraction, hindered metastatic progression, reduced

the expression of tumor markers and increased natural killer cell activity in all patients.[12,13] A polysaccharide component of mushrooms was reported to extend the survival of Japanese patients with estrogen-receptor-negative adenocarcinomas but not estrogen-positive breast tumors.[14] Other studies with MT have shown benefit against breast, lung and liver cancer although much less effectiveness against bone and stomach cancers, and acute type leukemia.[15] Despite mixed results, however, more than 90% of cancer patients have reported decreased side-effects from chemotherapy.

While previous results have been compelling, cancer research has largely focused on specialty or exotic mushrooms including shiitake, MT and reishi.[16] However, the two most popular mushrooms in the world are shiitake and white button (WB), with the former from the Far East and latter from the West.[17] A key, frequently reported protective mechanism exerted by mushrooms against cancer is the capacity to stimulate the immune system response where beta-glucan, a water-soluble polysaccharide, is presumed to activate certain immune cells and proteins that attack cancer, including macrophages, T-cells, natural killer cells, and interleukin-1 and -2.[6,18] Dietary mushrooms such as WB have also been shown to alter aromatase activity, an enzyme that functions in the conversion of androgens to proliferative estrogenic intermediates, which are closely linked to breast cancer development.[19,20] Aromatase is abundant in highly estrogen sensitive, proliferative tissues such as breast, uterus, vagina, bone, brain, heart and blood vessels.[21] Mushroom extracts, i.e. shiitake, contain other components such as lentinan and lectins, which are directly cytotoxic and cytostatic to tumor cells such as MCF-7 breast cancer cells.[22–24] WB mushrooms also contain bioactive antioxidants and anticarcinogenic substances including ergothioneine, selenium and polyphenols. Furthermore, studies have indicated that non-polysaccharide constituents in shiitake and oyster (OYS) mushrooms have biological activity against murine skin cancer and human prostate carcinoma cells.[14] Collectively, there are many bioactive agents in mushrooms that can exert beneficial effects co-operatively in systems with different cell types, i.e. immunity, or exhibit cytoxicity directly to cancer cells.

While much research has been conducted with specialty mushrooms, the most commonly consumed mushroom (90% of total consumption) in the USA is *Agaricus bisporus* or the common WB mushroom and its two other forms, portabella (PORT) and crimini (CRIM) or brown mushrooms. Thus, it would seem prudent to study both specialty and common mushrooms for beneficial effects on breast cancer. In this study, we analyzed markers of cellular proliferation, apoptosis and cytotoxicity/necrosis to determine and compare the beneficial effects of hot water extracts of both exotic or specialty mushrooms and commonly consumed dietary mushrooms on estrogen receptor-positive MCF-7 human breast cancer cells.

Methods

Chemicals

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), trypan blue, sodium pyruvate, trypsin and nicotinamide adenine dinucleotide (reduced) were obtained from Sigma Chemical Company (St Louis, MO, USA). Plasticware (plates and flasks) was purchased from Corning, Inc (Corning, NY, USA). All other chemicals and cell culture reagents were obtained from Sigma Chemical Company.

Cell culture

MCF-7 human breast cancer cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in flasks at 37°C in a humidified 5% CO_2 atmosphere. Cells were grown in minimum essential medium (MEM) medium with 1 mmol/L sodium pyruvate, 25 mmol/L sodium bicarbonate and 10% (v/v) fetal bovine serum. MEM media was further supplemented with 0.01 mg/mL bovine insulin, 1% penicillin (100 U/mL) and 1% streptomycin (100 μg/mL) (Sigma). Stock flasks were grown to ~70% confluency and routinely subcultured by trypsinization. Medium renewal was 2–3 times weekly.

For all experiments, cells were seeded and grown in 96-well plates (10^6 cells/mL; 10^5 cells/well) and T25 flasks (5×10^5 cells/mL; 10^6 cells/flask). At 70% confluency, medium was removed and cells were treated with complete MEM containing increasing concentrations (0.1–10.0% v/v) of mushroom extracts or the respective water vehicle control. After incubating for 24 h, medium was removed, and cells were washed twice with phosphate-buffered saline (PBS), observed, harvested and assayed for lactate dehydrogenase (LDH) release to determine an optimal concentration for subsequent experiments.

Preparation of mushroom extracts

Samples of mushrooms were collected from The Pennsylvania State University Mushroom Test Demonstration Facility (MTDF), The Pennsylvania State University Mushroom Research Center (MRC) and Modern Mushroom Farm, Inc (Toughkennamon, PA, USA) as shown in Table 1. Mushrooms were grown using the standard tray system under controlled conditions and were harvested at the optimum maturation stage with closed caps that were 2.0–2.5 inches in diameter. Mushrooms from the second break of the *A. bisporus* crops were tested and included brown

Table 1 Selected test mushrooms and sources

Mushroom species	Type of sample	Source
Agaricus bisporus	White button	Pennsylvania State University
Agaricus bisporus	Brown mushroom (Crimini)	Pennsylvania State University
Agaricus bisporus	Portabella	Pennsylvania State University
Lentinula edodes	Basidioma (Shiitake)	Modern Mushroom Farm, Inc
Grifola frondosa	Basidioma (Maitake)	Modern Mushroom Farm, Inc
Grifola frondosa	Basidioma (Maitake)	Pennsylvania State University
Pleurotus ostreatus	Basidioma (Oyster)	Modern Mushroom Farm, Inc

(CRIM) mushrooms, PORT mushrooms (mature, brown mushrooms with exposed hymenia) and the common WB mushrooms. Specialty mushrooms were harvested using standard mycology protocols and harvested on peak production days.

Harvested mushroom crops were randomly sampled, cleaned, sliced and stored at 0°C for 24 h. Samples were later freeze-dried (Virtis Freeze Dryer, Model 15 SRC-X; Virtis Genesis Co, Inc, Gardiner, NY, USA), ground to a fine powder and sieved through a 16 mesh screen. Mushroom powders were collected in sterile sample bags (Fisher Scientific, Pittsburgh, PA, USA) and stored in the dark at room temperature in desiccators.

Mushrooms were prepared as described previously and 10 g added to sterile plastic bottles containing 200 mL of distilled deionized water. Solutions were heated at 80°C for two hours followed by cooling at 25°C, centrifugation (1700*g*, 30 min), filtration through gauze, re-centrifugation of supernatant at 14,000*g* for 30 min and then filtration through 0.22 μm syringe filters. Solutions of sterile extracts (5% v/v) were prepared in complete MEM medium. Initial dose response experiments (0.6–30.0% [v/v]) were performed using a viability assay to determine the optimal concentration of vehicle, viz. water, to be used in this study. Five percent water was the highest concentration used that did not elicit adverse effects and was selected for subsequent experiments (data not shown).

Cellular proliferation by bromodeoxyuridine incorporation

Cellular proliferation was determined using a bromodeoxyuridine (BrdU) incorporation assay. Briefly, stock Brdu was diluted 1:2000 in MEM medium and added to supernatant in wells of control and treated cells two hours prior to harvest. After washing monolayers and fixing cells, an enzyme-linked immunosorbent assay was performed as described by the manufacturer (Trevigen, Gaithersburg, MD, USA) to measure DNA incorporation of BrdU. After addition of stop solution, samples were analyzed at 450 nm by spectroscopy.

Cell number by hemacytometry

After overnight incubation with media containing vehicle alone or containing mushroom extracts, monolayers were washed twice with PBS, trypsinized, neutralized with complete media, centrifuged and resuspended in sterile media. An aliquot (50 μL) of cell suspension was added to an equal volume of trypan blue and cells counted by light microscopy using a hemacytometer to determine viability according to standard protocols.[25] Dead cells, but not live cells, stained intensely with trypan blue and indicated cytotoxicity. Viability was determined using the following formula: % viability = ((live cells)/(dead cells + live cells)) × 100.

Mitochondrial activity by the MTT assay

Monolayers were incubated overnight with vehicle alone or containing mushoom extracts as described before, then washed and phenol red-free medium with MTT (5 mg/mL) was added for four hours at 37°C. Subsequently, plates were centrifuged for 10 min (1500*g* at 25°C) to pellet reduced precipitated MTT crystals. Conditioned medium was aspirated, isopropanol (0.1 mL per well) was added, samples were mixed (15 min at 25°C) to solubilize MTT and solutions were analyzed at 560 nm by spectroscopy.

Programmed cell death by terminal deoxynucleotidyl end labeling method

MCF-7 cells were incubated for 24 h as described above. Apoptosis was determined by the terminal deoxynucleotidyl end labeling method (TUNEL) as described by the manufacturer (Trevigen). Briefly, cells were pelleted by low-speed centrifugation, fixed in 37% (v/v) buffered formaldehyde and permeabilized. Endogenous peroxidase activity was quenched using 3% (v/v) hydrogen peroxide in methanol and cells were incubated for one hour at 37°C with labeling reaction mix (terminal deoxynucleotidyl transferase, Mn^{2+}, and biotinylated dNTPs), followed by streptavidin horseradish peroxidase. Apoptosis was determined by color development using TACS sapphire substrate and measured at 450 nm after addition of stop reagent (2 N HCl).

Necrosis and cytotoxicity by LDH release

MCF-7 cells were incubated for 24 h in 96-well plates as described above. After low-speed centrifugation, aliquots of supernatant (100 μL) were transferred to a new 96-well plate with an ultraviolet transparent bottom (Costar, Corning, NY, USA). To this aliquot, 85 μmol/L reduced NADH in potassium phosphate buffer (100 mmol/L; pH 7.5) was added and plates were placed on a heated (37°C) microplate reader set to kinetics mode. To initiate the enzymatic reaction, an aliquot of 7 mmol/L sodium pyruvate was added, plates were shaken for 3 s and readings were taken at 340 nm every 15 s for 10 min. For measurements of total cellular activity, an aliquot (100 μL) of sodium dodecyl sulfate (0.1% v/v) was added to monolayers for 10 min at 25°C before proceeding with the LDH assay as described above. Rates of NADH oxidation were determined and enzymatic activity was calculated. Data are expressed as the percent of the vehicle control. LDH activity in medium and cells was determined and expressed as: % release = ([LDH activity in media]/[LDH activity in media + LDH activity in cells]) × 100.

Analysis of total polyphenols

The Folin-Ciocalteau method was used to quantify total polyphenols in samples of aqueous mushroom extracts as described previously.[26,27] Three milliliters of water, 200 μL Folin-Ciocalteau reagent and 200 μL of diluted sample or standard (gallic acid) were added to test tubes and mixed well by gentle vortexing (3 s; low speed). Tubes were incubated at room temperature (25°C) for 10 min followed by addition of 600 μL of 20% Na_2CO_3. Tubes were then incubated in a water bath at 40°C for 20 min followed by immediately cooling in an ice bath to room temperature

25°C (30 min). After cooling, samples were analyzed at 755 nm. Purified gallic acid was used as the external standard.

Statistical analysis

Statistical data were analyzed using SPSS (version 15.0 for Windows) and expressed as means ± SEM. Data were tested for normality; non-parametric testing was used to analyze non-normal data. One-way analysis of variance was used to determine significant differences between treatment groups and control cultures. Tukey's *post hoc* analysis was used to determine significant differences between responses to mushroom extracts. Significance was determined at $P \leq 0.05$. Experiments were performed 2–3 times with three to eight replicates for each assay.

Results

BrdU incorporation is a routinely used non-radioisotopic assay of cellular proliferation where a brominated nucleic acid, deoxyuridine, is incorporated into replicating DNA prior to cellular proliferation. Cellular proliferation was set at 100% for control cultures. All test mushrooms significantly ($P < 0.05$) suppressed BrdU incorporation up to 33% (Figure 1). Extracts of MT and OYS appeared to be the most potent with 25% and 33% reductions, respectively, followed by equivalent, significant reductions of 15% by CRIM, PORT and WB. The results indicate that all test mushrooms significantly reduced BrdU incorporation and presumably cellular proliferation.

The capacity for cells to reduce water-soluble MTT to its precipitable formazan product has been previously used as an indicator of both cellular proliferation and cytotoxicity since the assay is based on the presence and function of succinate dehydrogenase located in the mitochondrion. To corroborate the BrdU incorporation data, we tested the effects of test mushrooms on cellular capacity to reduce MTT. We observed no significant differences in MTT reduction when MCF-7 cells were incubated with the five test mushrooms (Figure 2). However, we did note that cells preincubated with OYS displayed a 20% reduction in the capacity to metabolize MTT although this effect was not statistically significant.

Given the results of the MTT assay, we next determined by hemacytometry actual total cell numbers in each flask after overnight incubation with control medium or media containing test mushroom extracts. The average cell number was $6–7 \times 10^6$ cells per flask and there were no significant differences between the cultures preincubated with test mushrooms or the control cultures receiving water only (Figure 3). These results corroborated the MTT data that cell number was not significantly reduced.

Given the disparity in results between BrdU incorporation and cytotoxicty and viability assays, we assessed the cellular release of LDH into cell culture supernatant as an indicator of cytotoxicity and necrosis. LDH is a large intracellular cytoplasmic enzyme that leaks into the medium supernatant only when there is a compromise, or substantial leakiness, of the plasma membrane. The MT extract significantly increased LDH release to 9% when compared with control cultures, which displayed 4% LDH leakage (Figure 4). Extracts of CRIM, PORT, OYS and WB were not significantly different from one another or control cultures. CRIM was not different from CTL or MT, suggesting an intermediate degree of release. WB and MT, extracts were not significantly different from one another. The results indicate that MT induced significant cytotoxicity and subsequent necrosis, whereas other test mushrooms were not cytotoxic under these experimental conditions.

After testing for cellular proliferation using BrdU incorporation and cytotoxicity/necrosis using several different

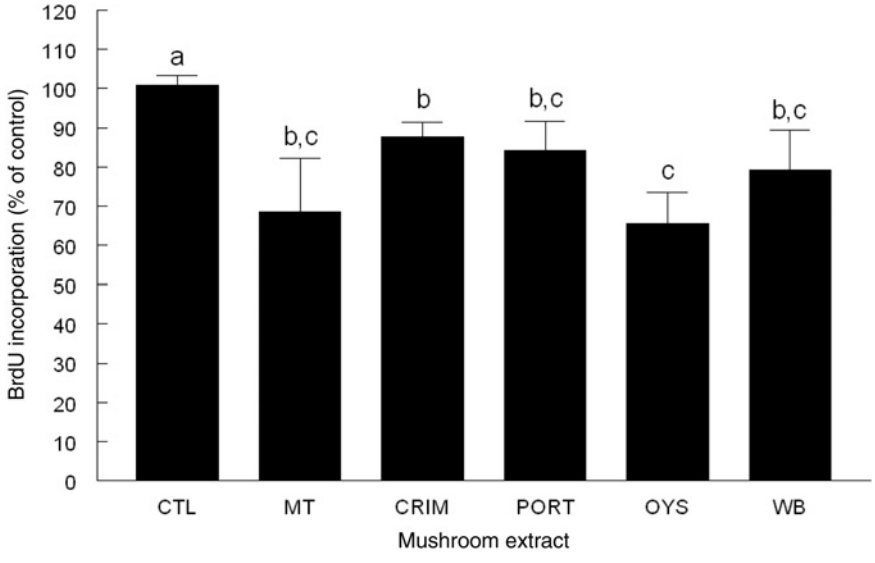

Figure 1 Effect of hot water extracts (5% v/v) of dietary mushrooms or water vehicle alone on cellular proliferation in MCF-7 human breast cancer cells incubated for 24 h. Proliferation was determined by bromodeoxyuridine (BrdU) incorporation into replicating DNA of cells. Data are expressed as percent of control ± SEM. Bars with differing letters are significantly ($P < 0.05$) different from one another. CTL, control; MT, maitake; CRIM, crimini; PORT, portabella; OYS, oyster; WB, white button

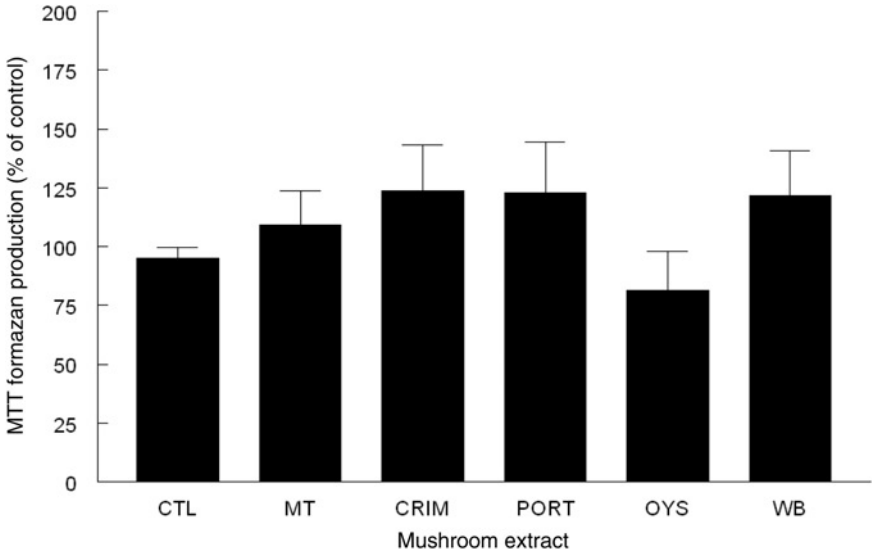

Figure 2 Effect of hot water extracts (5% v/v) of dietary mushrooms or water vehicle alone on cytotoxicity and cell number in MCF-7 human breast cancer cells incubated for 24 h as determined by MTT reduction. Data are expressed as percent of control ± SEM. CTL, control; MT, maitake; CRIM, crimini; PORT, portabella; OYS, oyster; WB, white button; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)

assays, we next focused on programmed cell death or apoptosis. Four of the five mushrooms (CRIM, PORT, OYS and WB) increased apoptosis by 15–20% but this effect was not statistically significant although perhaps potentially biologically relevant (Figure 5). Extracts of MT mushrooms did, however, significantly increase apoptosis by 45% above the response of control cultures. Collectively, there were trends for increased apoptosis by all mushroom extracts, with MT clearly exerting a proapoptotic effect under these experimental conditions.

Given the plethora of bioactive agents in dietary mushrooms, we tested the total polyphenol content as one representative class of molecules. Aliquots of samples after preparation of the extracts were analyzed by the Folin method as described previously.[26] OYS and MT had the highest amounts of total polyphenols (Figure 6), whereas PORT and CRIM had intermediates levels and WB had the lowest amount of total polyphenols in the final extract solution.

Discussion

Only a small percentage (<10%) of dietary mushrooms have been rigorously evaluated for antitumor properties in the context of cancer treatment. Moreover, research has focused primarily on exotic or specialty mushrooms including shiitake, MT and reishi. However, the most commonly consumed mushroom in the USA is *A. bisporus* or the WB

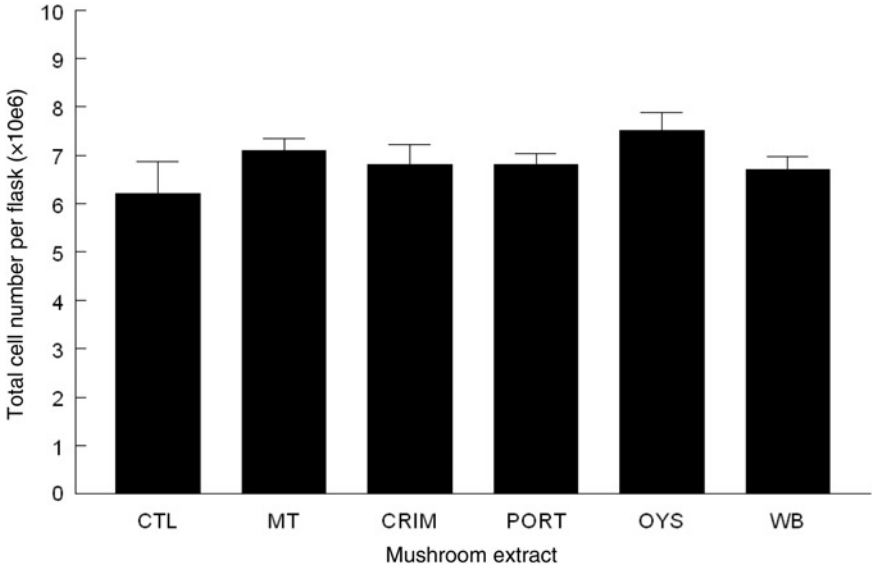

Figure 3 Effect of hot water extracts (5% v/v) of dietary mushrooms or water vehicle alone on total cell numbers of MCF-7 human breast cancer cells incubated for 24 h. Cell numbers were determined by hemacytometry and are expressed as total cell number per T25 flask. CTL, control; MT, maitake; CRIM, crimini; PORT, portabella; OYS, oyster; WB, white button

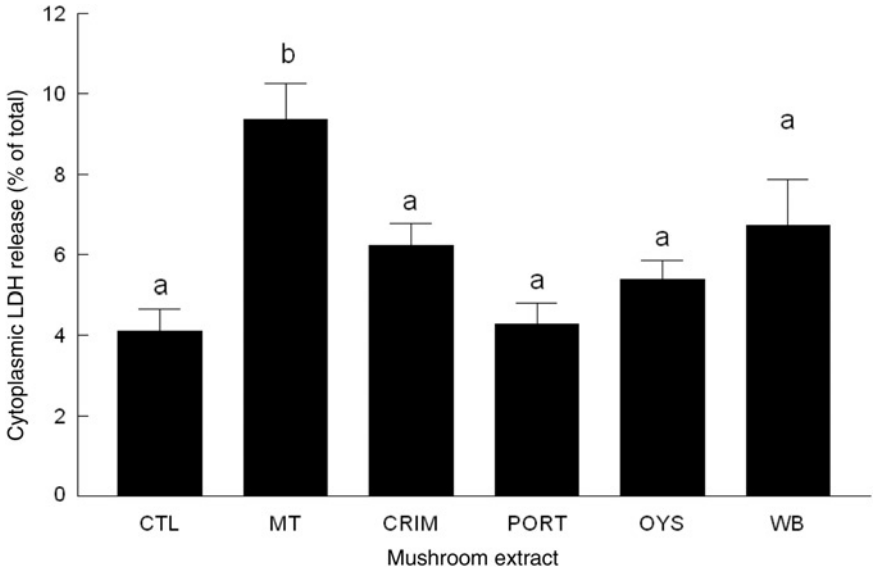

Figure 4 Effect of hot water extracts (5% v/v) of dietary mushrooms or water vehicle alone on necrosis in MCF-7 human breast cancer cells incubated for 24 h. Necrosis was determined by lactate dehydrogenase (LDH) release from the cytoplasm to cell culture supernatant. Data are expressed as percent release calculated as: % release = ((LDH activity in media)/(LDH activity in media + LDH activity in cells)) × 100. Data are expressed as percent of control ± SEM. Bars with differing letters are significantly ($P < 0.05$) different from one another. CTL, control; MT, maitake; CRIM, crimini; PORT, portabella; OYS, oyster; WB, white button

mushroom. As a result, our goal in this study was to test and compare the effects of several mushroom types, including both commonly consumed and exotic fungi, on several cellular processes involved in the elaboration of breast cancer. Our results clearly indicate that all test mushrooms (MT, CRIM, PORT, OYS and WB) could significantly inhibit to a similar magnitude the cellular proliferation of human breast cancer cells. MT, however, did appear to be the most potent and consistent in that extracts also significantly induced apoptosis and cytotoxicity whereas only trends were noted for the other mushroom extracts.

Many diverse species of mushrooms and their respective bioactive agents have demonstrated the capacity to inhibit cellular proliferation of human breast cancer cells *in vitro*. For example, extracts of PORT, shiitake and OYS suppressed proliferation of both estrogen-receptor(ER)-positive MCF-7 and ER-negative MDA-MD-231 human breast cancer cells without affecting proliferation of normal cells.[28] Hot water extracts of *Coprinellus* sp., *Coprinus comatus* and *Flammulina velutipes* also significantly inhibited cellular proliferation of MCF-7, MDA-MB-231 and BT-20 cells.[14] Isolated lectins from *Pholiata adipose* and *Inocybe umbrinella* exerted antiproliferative activity toward MCF-7 and

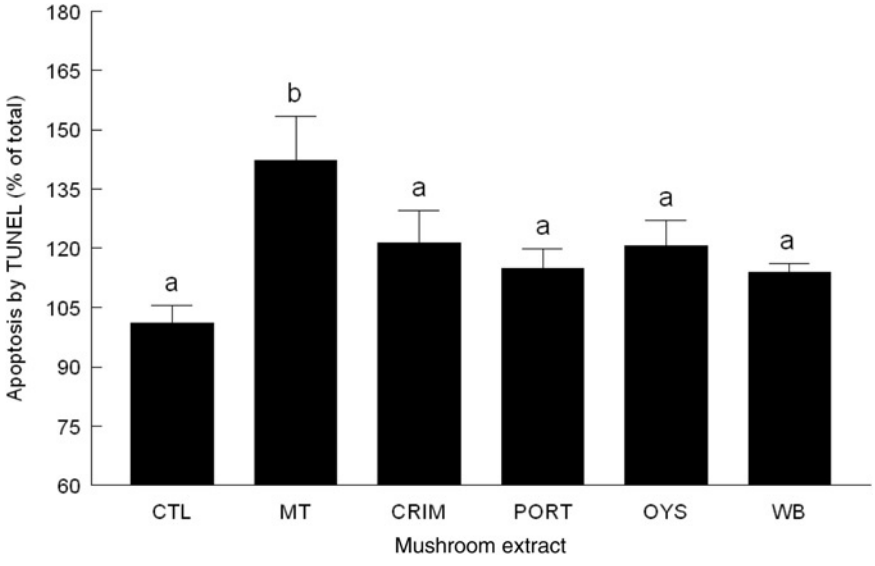

Figure 5 Effect of hot water extracts (5% v/v) of dietary mushrooms or water vehicle alone on apoptosis, or programmed cell death, in MCF-7 human breast cancer cells incubated for 24 h. Apoptosis was determined by terminal deoxynucleotidyl end labeling method (TUNEL). Data are expressed as percent of control ± SEM. Bars with differing letters are significantly ($P < 0.05$) different from one another. CTL, control; MT, maitake; CRIM, crimini; PORT, portabella; OYS, oyster; WB, white button

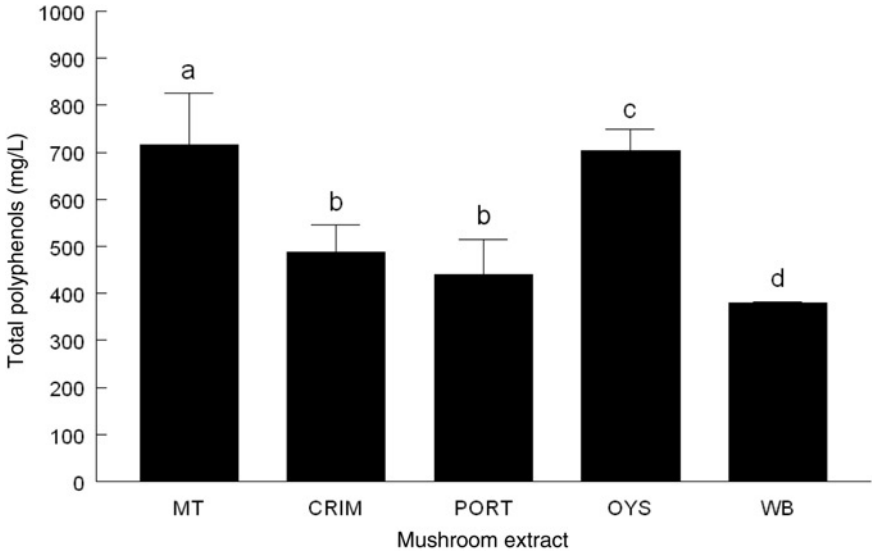

Figure 6 Quantification of total polyphenol concentrations in hot water extracts of dietary mushrooms. Total polyphenols were determined using the Folin method and expressed as mg gallic acid equivalents/L using gallic acid as an external standard. Bars with differing letters are significantly ($P < 0.05$) different from one another. CTL, control; MT, maitake; CRIM, crimini; PORT, portabella; OYS, oyster; WB, white button

HepG2 human hepatoma cells with IC_{50}s in the range of 1.9–7.5 μmol/L.[29,30] An isolated *A. bisporus*, or WB, lectin inhibited incorporation of ^{3}H-thymidine into DNA by 50% in MCF-7 cells and by 87% in HT29 colon cancer cells, 16% in Caco-2 colon cancer cells and by 55% in rat mammary fibroblasts after 24-h incubation. This is in agreement with our observations since we noted a 33% reduction in BrdU incorporation after 24-h incubation using MCF-7 cells. Moreover, this effect was noted in both specialty and commonly consumed mushrooms at a similar magnitude. This is in contrast to the report by Gu and Leonard[14] who tested 36 different mushroom extracts and found up to a 10-fold difference in the IC_{50} for growth inhibiton. Specific bioactive agents such as hispolon and marmorin, proteins isolated from *Phellinus linteus* and *Hypsizigus marmoreus*, respectively, can also significantly inhibit MCF-7 breast, bladder and liver cancer cell proliferation and induce apoptosis.[31,32] Conjugated metabolites such as sulfated water-soluble alpha 1-3 D-glucans of *Lentinus edodes*, or shiitake, have also been shown to exert potent antiproliferation action (by 52%) on MCF-7 cells.[33] Clearly, numerous species of mushrooms delivered as whole mushroom extracts or isolated bioactive components, or their metabolites, can inhibit cellular proliferation, suggesting that this is a key mechanism of action.

There have been many proposed mechanisms for the inhibitory action of mushrooms including immune system boosting and cell signaling inhibition. For example, studies show that shiitake has tumor-inhibitory effects on proliferation possibly through reduction in natural killer cell cytotoxicity in rodent studies.[34] We have previously shown using *in vitro*, *ex vivo* and rodent models that dietary mushrooms including WB could increase cytokine production and enhance immune function of antitumor cells.[6] Other *in vitro* studies have shown that bioactive agents such as ganaderic acid from *Ganoderma lucidum*, or reishi, suppressed cellular proliferation and invasive behavior in MDA-MD-231 human breast cancer cells by the inhibition of transcription factors activator protein 1 and nuclear factor κB.[35] Additionally, *P. linteus* inhibited anchorage-dependent cellular proliferation and anchorage-independent colony formation in the same cell line mediated via AKT signaling.[36] Clearly, dietary mushrooms can function through many cellular mechanisms, two of which involve the immune system and cell signaling when using multicellular, multisystem models.

Mechanistically, dietary mushrooms may also induce cell cycle arrest in cancer cells. In one study, *Pleurotus ostreatus* (OYS), compared with PORT, shiitake and enoki, was the most potent of tested mushrooms in reducing growth of MCF-7 cells, which was associated with cell cycle arrest in the G0/G1 phase.[28] In a second study, Sliva *et al.*[28] confirmed that OYS mushroom extracts potently inhibited proliferation by inducing cell cycle arrest at the G0/G1 phase in MCF-7 cells. Alternately, an extract of *A. blazei* inhibited cell growth in a dose-dependent manner by arrest of cells in the G2/M phase and induced apoptosis.[37] Aqueous extracts of I'm-Yunity, a mushroom extract from *Coriolus versicolor*, also inhibited cellular proliferation and induced apoptosis in HL-60 and U-937 cells by disruption of both the G1/S and G2/M phases of cell cycle progression.[38] This is consistent with our results where proliferation was not altered as indicated by the MTT assay and cell counts, but DNA replication clearly was decreased, suggesting an effect on the cell cycle. That is, during the G1/S transition, cells are prepared for cellular proliferation through preemptive DNA replication, but if cells are arrested at this checkpoint, viz. G0/S, then DNA replication will be reduced or prevented. Our results indicate a ~30% reduction by the test mushrooms on mitogenesis, as measured by BrdU incorporation, indicative of a block in DNA replication.

Four of the five mushrooms increased apoptosis by 15–20% but this was not statistically significant under our experimental conditions. It is likely that a longer time of exposure of MCF-7 cells to mushroom extracts would have

markedly increased apoptosis. For example, Zhang *et al.* purified water-soluble beta-glucan from mycelium of *Poria cocos* and incubated MCF-7 cells with 12.5–400 μg/mL for 72 h, which significantly reduced proliferation and viability and dose-dependently induced apoptosis. Up to 90% of the cells accumulated in the G1 phase after 72 h (G1 arrest).[39] Reishi has also been shown to induce both cell cycle arrest at the G2/M checkpoint and apoptosis in PC3 cells.[40] Specifically, the MT D-fraction at 480 μg/mL increased apoptotic cell death to >95% after 24-h incubation whereas reishi and *A. blazei* reduced proliferation by ~50% and ~5%, respectively, demonstrating variability in potency and, perhaps, mushroom-specific responses.[23] We noted in these studies that MT extract appear to be the most potent and consistent compared with other test mushrooms in significantly reducing BrdU incorporation, increasing apoptosis and increasing LDH release. Compelling evidence indicates that MT has potent antitumor and anticancer activity due to the presence of water-soluble beta 1,6-glucans associated with the high molecular weight D-fraction, which is an acid-insoluble, protein-bound polysaccharide that is hot water extractable.[13]

Lastly, we assessed cytotoxicity by evaluating the cytoplasmic release of the large cytoplasmic protein LDH into cell culture supernatant. MT extracts significantly increased LDH release to twice that of control cultures, indicating direct disruption of plasma membrane function and subsequent cytotoxicity. Extracts of CRIM, PORT, OYS and WB were not significantly different from one another or control cultures demonstrating a lack of cytotoxic effect to the plasma membrane. *A. bisporis*, or WB mushroom, has been shown to be non-cytotoxic although inhibiting cell proliferation.[41] Other studies using isolated lectins from mushrooms also showed lack of cytotoxicity to HT29, MCF-7 and RAMA27 cells as measured by trypan blue exclusion but inhibition of growth or proliferation.[41]

The potent action of MT compared with the other mushrooms may be explained, in part, by the extraction methodology. We extracted test mushrooms at 80°C for two hours from 10 g of powdered material. Since extraction of polysaccharides requires boiling temperatures and MT has high levels of water-soluble beta-glucans, it is likely that more was extracted from MT than from the other mushrooms.[14] The amount of starting material, the geographic origin of the raw mushroom material, i.e. MT, and the composting procedures could also enrich dry material with bioactive agents potentially conferring additional potency. In contrast to others, we did not evaporate the extraction solvent to dryness or concentrate the bioactive components with reconstitution but used the extract as prepared. Briefly, we started with 10 mg whole-mushroom powder in 200 mL water producing a 0.05 mg/mL solution, which was further diluted to 5% (v/v) resulting in a 2.5 μg/mL concentration with an actual addition of mushroom to cultures at 250 ng per well. In a report by Gu and Leonard[14] using MCF-7 cells, the IC_{50}s for growth inhibition ranged from 120 to 450 μg/mL. Furthermore, a concentration of 100 μg/mL had no effect on viability or growth inhibition, but increasing the concentrations to 200 and 400 μg/mL significantly decreased cellular proliferation and induced apoptosis. In fact, extracts of *Coprinellus* sp. and *F. velutipes* induced a rapid, early apoptosis within two hours and late apoptosis as shown by DNA fragmentation (measured by TUNEL) within five hours using markedly higher concentrations than used in our study. In addition to potentially differing extraction efficiencies, dose clearly is important. Although our calculated mushroom concentrations were low, we were, nonetheless, able to detect a beneficial effect and ultimately compare the relative potency of test mushrooms.

The relative bioactive agent composition of MT may also explain, in part, the relative potency compared with other test mushrooms. The active polysaccharides in MT are largely beta-glucans and vary considerably in concentration in mushrooms.[42] Ergothioneine, a unique antioxidant that is particularly prevalent in mushrooms, is highest in specialty mushrooms such as MT and OYS compared with the other mushrooms. As reported by DuBost *et al.*[27] for the test mushrooms used here, ergothioneine levels were 1.1, 0.4, 0.5, 2.6 and 0.2 mg/g dry weight for MT, CRIM, PORT, OYS and WB, respectively, with MT and OYS having the highest amounts. Recall that both MT and OYS significantly reduced BrdU incorporation and OYS markedly reduced, although non-significantly, MTT reduction by 20%. We were also interested in the total polyphenol content of mushrooms due to the prevalence and demonstrated bioactivity of this family of compounds. The amount of polyphenols per dry weight has previously been reported by DuBost *et al.*[27,43] for the test mushrooms used here to be 4.2, 9.9, 10.6, 4.3 and 8.0 mg gallic acid equivalents/g for MT, CRIM, PORT, OYS and WB, respectively, with MT unexpectedly being one of the lowest. However, we tested the concentrations of identical mushroom powders after our extraction process and found that the levels were higher in MT and OYS compared with the other test mushrooms (Figure 6). This suggests either concerns with stability during storage and preparation of the extract or considerable variability in extractability under the reported experimental conditions.

Only a small percentage (<10%) of dietary mushrooms have been extensively tested in models of breast cancer. Moreover, research has focused primarily on exotic or specialty mushrooms including shiitake, MT and reishi instead of the most commonly consumed mushroom in the USA, which is *A. bisporus* or the WB mushroom. Our results clearly indicate that all test mushrooms can significantly inhibit to a similar magnitude the cellular proliferation of human breast cancer cells. MT, however, did appear to be the most potent and consistent in that extracts also significantly induced apoptosis and cytoxicity where only trends were noted for the other mushroom extracts. Collectively, the data support that dietary mushrooms, both common and specialty, can protect against breast cancer and should be part of a healthy diet in addition to high fruit and vegetable consumption. Clearly, research focusing on the pharmacological value and identification of mechanisms of action of dietary mushrooms against breast cancer is warranted.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; KRM and SKB conducted

the experiments; KRM supplied reagents and materials and wrote the manuscript.

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