

***Pleurotus ostreatus* inhibits proliferation of human breast and colon cancer cells through p53-dependent as well as p53-independent pathway**

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Abstract. In spite of the global consumption of mushrooms, only two epidemiological studies demonstrated an inverse correlation between mushroom intake and the risk of cancer. Therefore, in the present study we evaluated whether extracts from edible mushrooms *Agaricus bisporus* (portabella), *Flammulina velutipes* (enoki), *Lentinula edodes* (shiitake) and *Pleurotus ostreatus* (oyster) affect the growth of breast and colon cancer cells. Here, we identified as the most potent, *P. ostreatus* (oyster mushroom) which suppressed proliferation of breast cancer (MCF-7, MDA-MB-231) and colon cancer (HT-29, HCT-116) cells, without affecting proliferation of epithelial mammary MCF-10A and normal colon FHC cells. Flow cytometry revealed that the inhibition of cell proliferation by *P. ostreatus* was associated with the cell cycle arrest at G0/G1 phase in MCF-7 and HT-29 cells. Moreover, *P. ostreatus* induced the expression of the tumor suppressor p53 and cyclin-dependent kinase inhibitor p21^(CIP1/WAF1), whereas inhibited the phosphorylation of retinoblastoma Rb protein in MCF-7 cells. In addition, *P. ostreatus* also up-regulated expression of p21 and inhibited Rb phosphorylation in HT-29 cells, suggesting that that *P. ostreatus* suppresses the proliferation of breast and colon cancer cells via p53-dependent as well as p53-independent pathway. In conclusion, our results indicated that the edible oyster mushroom has potential therapeutic/preventive effects on breast and colon cancer.

Introduction

Cancer is a major public health problem in the United States and other developed countries. Currently, one in four deaths

in the United States is related to cancer. Breast cancer in women and colon cancer in men and women continue to be the most common fatal cancers (1). Therefore, modifications of diet and lifestyle offer measures for reducing the risk of developing breast and colon cancer. Carcinogenesis is a complex, multistep process that progresses over many years. Since it is exceptionally difficult to cure malignant tumors, cancer prevention may be a more effective strategy to control and ultimately, overcome cancer. A promising and important group of potential cancer preventive agents are those derived from natural products, particularly dietary substances because of their low toxicity and apparent benefit in other chronic diseases (2).

Edible mushrooms are a valuable source of biologically active compounds (3). The use of mushrooms with potential therapeutic properties raises global interest from the scientific and clinical community based on two main reasons. First, mushrooms demonstrate their efficiency against numerous diseases and metabolic disturbances as serious as cancer or degenerative diseases. These therapeutic effects seem to lay multiple complex pharmacological actions on different cellular and molecular targets (4). The most significant medicinal effects of mushrooms and their metabolites that have attracted the attention of the public is their antitumor properties (5,6). The medicinal use of edible mushrooms extracts seems to be a more natural, less expensive approach and in general involves minimal unwanted side effects. Moreover, purified bioactive compounds derived from edible mushrooms might be a potentially important new source of anticancer agents. The oyster mushroom (*Pleurotus ostreatus*) is one of the widely cultivated edible mushrooms (7). Several properties have been associated with this mushroom, including antitumor (8), hypocholesterolemic, antiatherogenic, and antioxidative activities (9,10). Moreover, it has also been reported that the oyster mushroom can increase the immune activity of animals (11). In addition, it has been shown that crude oyster extract has a cytotoxic effect on PC-3 cells (12) and aqueous polysaccharide extract has anti-proliferative and pro-apoptotic effects on HT-29 cells (13). However, the molecular mechanism(s) responsible for the inhibitory effects and modulation of cell cycle by *P. ostreatus* on the breast and colon cancer cells has not been fully elucidated.

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In the present study, we examined the effect of edible mushrooms on the proliferation of human breast and colon cancer cells. Based on our results we propose the mechanism by which *P. ostreatus* inhibits growth of breast and colon cancer cells.

Materials and methods

Materials. Edible mushrooms (*Agaricus bisporus*, *Flammulina velutipes*, *Lentinula edodes* and *Pleurotus ostreatus*) were purchased from a local market (Meijer) in Indianapolis, USA.

Preparation of methanol extracts. The mushrooms were ground using a food mixer (Osterizer blender, Sunbeach Products Inc., Boca Raton, FL, USA). This smashed biomass (100 g) was suspended in 400 ml of absolute methanol and incubated for 48 h at 200 rpm and 37°C. The suspension was filtered on Whatman No. 3 paper to remove the biomass. This procedure was repeated twice. The supernatant was concentrated in a rotary evaporator at 50°C under reduced pressure. The resulting dried biomass was dissolved in sterile distilled water to make stocks 50 mg/ml and stored at 4°C.

Cell cultures. Human cell lines (MDA-MB-231, MCF-7, MCF-10A, HCT-116, HT-29 and FHC) were obtained from ATCC (Manassas, VA, USA). The human breast cancer cell lines (MDA-MB-231 and MCF-7) and human colon cancer cell lines (HCT-116 and HT-29) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin (50 U/ml), streptomycin (50 U/ml) and 10% fetal bovine serum (FBS). The human mammary epithelial cell MCF-10A cells were maintained in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 5% donor horse serum, 20 ng/ml EGF (Upstate, Lake Placid, NY, USA), 10 µg/ml insulin (Sigma, St. Louis, MO, USA), 100 ng/ml cholera toxin (Bioscience, La Jolla, CA, USA), 0.5 µg/ml hydrocortisone (Sigma), 50 U/ml penicillin and 50 U/ml streptomycin. The normal human fetal colon cells (FHC) were cultured in a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle's medium's containing 25 mM HEPES (Sigma), 10 ng/ml cholera toxin (Bioscience, La Jolla, CA, USA), 5 µg/ml insulin (Sigma), 5 µg/ml transferrin (Bioscience), 100 ng/ml hydrocortisone (Sigma), and supplemented with 10% FCS. The cultures were maintained at 37°C in 5% CO₂ and 95% humidity.

Cell proliferation assay. The anti-proliferative activity of mushroom extracts was evaluated by the tetrazolium salt method according to the manufacturer's instructions (Promega, Madison, WI, USA). All cell lines were cultured in a 96-well plate and treated at indicated times with mushroom extracts (0-1.0 mg/ml). At the end of the incubation period, the absorption was determined with a plate reader at 570 nm as previously described (14).

Morphological examination of cancer cells. Cancer cells were grown in 60-mm tissue culture dish and treated with *P. ostreatus* extract (0-1.0 mg/ml). Cells were examined under a fluorescent microscope (Olympus, Tokyo, Japan) and

pictures were taken using a Magna-Fire digital camera (Optotronics, Goleta, CA, USA) for analysis.

Cell cycle analysis. MCF-7 cells (1x10⁶) and HT-29 cells (1x10⁶) were seeded and after 24 h treated with *P. ostreatus* extract (1.0 mg/ml) for the indicated period (0-48 h). Cells were harvested by trypsinization, washed with Dulbecco's phosphate-buffered saline, and resuspended in propidium iodide (50 µg/ml). Cell cycle analysis was performed on a FACStarPLUS flow cytometer (Becton-Dickinson, San Jose, CA, USA) as previously described (15).

DNA microarrays. MCF-7 and HT-29 cells were treated for 24 h with vehicle or 1 mg/ml of *P. ostreatus* extract and total RNA isolated by RNeasy Mini Kit (Qiagen, Germantown, MD, USA). This RNA was used for the evaluation of expression of cell cycle regulatory genes with Cell Cycle Oligo GEArray according to the manufacturer's protocol (SABiosciences, Frederick, MD, USA). GEArray Expression Analysis Suite Software was used for the data analysis.

Western blot analysis. MCF-7 and HT-29 (1x10⁶) were treated with *P. ostreatus* extract (0-1.0 mg/ml) for 24 h and whole cell extracts were prepared as previously described (14). Equal amounts of proteins (15 µg/lane) were separated on 4-12% or 12% SDS-PAGE (Invitrogen, Carlsbad, CA, USA) and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membrane was incubated with the corresponding primary antibodies diluted 1:1000 in blocking solution, as follows: anti-p21, anti-Rb and anti-pRb (Cell Signaling, Danvers, MA, USA), anti-PCNA and anti-p53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The expression of each protein was detected by the ECL Western blotting detection system (Amersham Biosciences, Buckinghamshire, UK).

Densitometric analysis. Western blot autoradiograms were scanned with HP-Scanjet 550c. The optical densities of proteins were quantified and analyzed by the UN-SCAN-IT software (Silk Scientific, Orem, UT, USA).

Results

***P. ostreatus* inhibits the proliferation of breast and colon cancer cells.** In order to evaluate the effect of edible mushrooms on the growth of breast and colon cancer cells, we prepared methanol extracts from fruiting bodies of *Agaricus bisporus*, *Flammulina velutipes*, *Lentinula edodes* and *Pleurotus ostreatus*. As seen in Fig. 1, extracts from *A. bisporus*, *F. velutipes*, and *L. edodes* exerted little effect on proliferation, whereas the extract from *P. ostreatus* markedly decreased cell proliferation of MCF-7 as well as HT-29 cells. To investigate whether this effect is specific for cancer cells, we treated breast cancer cells (MDA-MB-231, MCF-7) and colon cancer cells (HCT-116, HT-29) with increasing concentrations of *P. ostreatus* (0-1.0 mg/ml) for 24 and 48 h. As seen in Fig. 2A, *P. ostreatus* suppressed the proliferation of highly-invasive breast cancer MDA-MB-231 cells and poorly-invasive breast cancer MCF-7 cells (Fig. 2B) in dose- and time-dependent manner. However, no significant

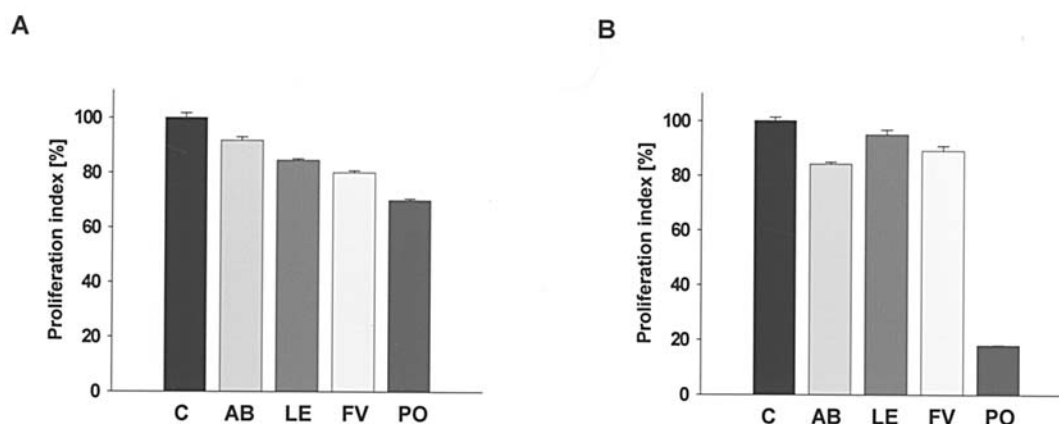


Figure 1. Effects of edible mushrooms on the proliferation of breast cancer and colon cancer cells. (A) MCF-7 and (B) HT-29 cells were treated for 24 h with 1 mg/ml of mushroom extract and proliferation was assessed as described in Materials and methods. Data are the mean \pm SD of triplicate determinations. C, Control; AB, *Agaricus bisporus*; LE, *Lentinus edodes*; FV, *Flammulina velutipes* and PO, *Pleurotus ostreatus*.

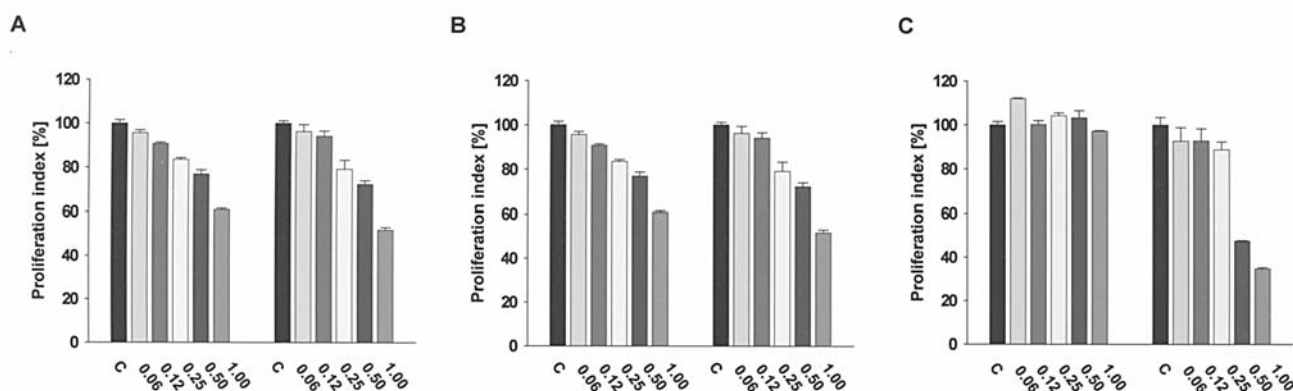


Figure 2. Effects of *P. ostreatus* on the proliferation of breast cancer and mammary epithelial cells. (A) MDA-MB-231, (B) MCF-7, (C) MCF-10A cells were treated with *P. ostreatus* (0-1.0 mg/ml) for 24 and 48 h. Proliferation was assessed as described in Materials and methods. Data are the mean \pm SD of triplicate determinations. Similar results were received in at least two additional experiments.

effect on non-tumorigenic breast epithelial cells MCF10A (Fig. 2C) was observed. In addition, *P. ostreatus* inhibited the proliferation of highly-invasive colon cancer HCT-116 cells (Fig. 3A) and poorly-invasive colon cancer HT-29 cells (Fig. 3B) in a dose- and time-dependent manner. In contrast, proliferation of normal colon cells FHC was not affected by treatment with *P. ostreatus* (Fig. 3C). These results suggest that *P. ostreatus* suppresses proliferation of breast cancer cells and colon cancer cells without significant effect on proliferation of normal human mammary and colon cells.

Effect of *P. ostreatus* on changes in cell morphology and cell cycle phase distribution. To further investigate the growth modulatory effects of *P. ostreatus* we examined the changes in MCF-7 and HT-29 cell morphology. Typically, MCF-7 cells grow in a flattened, star shape. However, *P. ostreatus* induced morphological alterations resulting in the elongated shape of MCF-7 cells. Moreover, some of the cells were lifted and also grew in a noticeably more disperse manner (Fig. 4). HT-29 cells grown in a typical goblet-like shape, reflecting propensity for adherence to each other as cells proliferate. *P. ostreatus* treatment of HT-29 cells changed the

cell form to a rounder shape and some cells were lifted from attachment (Fig. 4). In order to investigate the mechanism by which *P. ostreatus* inhibits growth of breast and colon cancer cells, we analyzed cell cycle distribution by flow cytometry. We found that treatment with *P. ostreatus* (1 mg/ml) caused cell cycle arrest at G0/G1 phase as indicated by the increased amount of MCF-7 and HT-29 cells at G0/G1 from 21% (0 h) to 60% (48 h) for MCF-7 (Fig. 5A) and from 26% (0 h) to 66% (48 h) for HT-29, respectively (Fig. 5B).

Effects of *P. ostreatus* on expression of cell cycle gene expression using cDNA array analysis. To identify genes responsible for the anti-proliferative effects of *P. ostreatus*, we used cDNA microarray analysis. This array profiles the expression of 112 genes key to cell cycle regulation such as cyclins, cyclin-dependent kinase (CDK) inhibitors, CDK phosphatases, CDK kinases and genes essential for the DNA damage and mitotic spindle checkpoints. In these experiments, MCF-7 and HT-29 cells were treated with *P. ostreatus* as described in Materials and methods. As seen in Table I, *P. ostreatus* induced expression of p21, p53, p27, p19 and down-regulated expression of CDK4, CDK6, Ki67, E2F

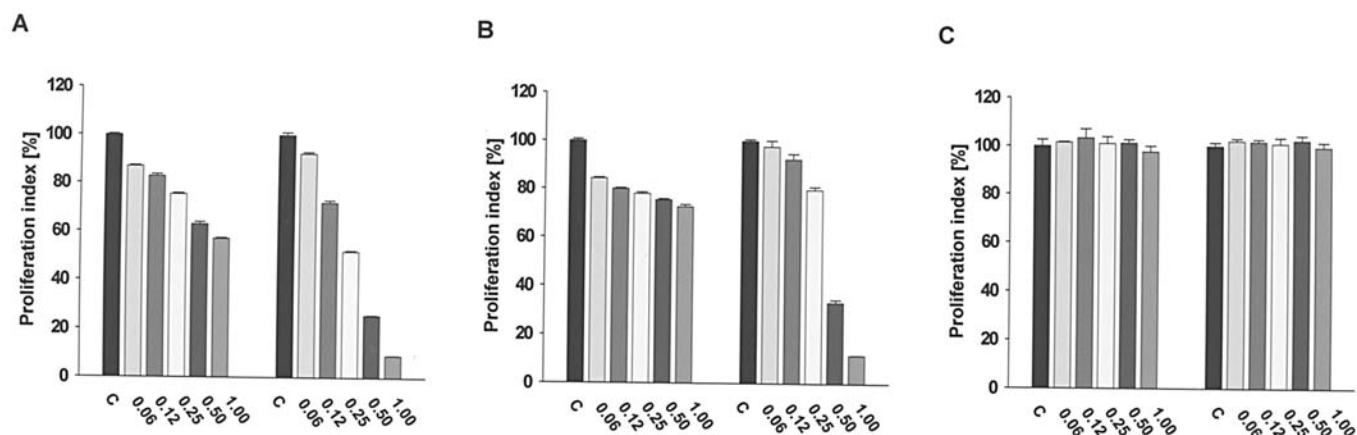


Figure 3. Effects of *P. ostreatus* on the proliferation of colon cancer and normal colon cells. (A) HCT-116, (B) HT-29, (C) FHC cells were treated with *P. ostreatus* (0-1.0 mg/ml) for 24 and 48 h. Proliferation was assessed as described in Materials and methods. Data are the mean \pm SD of triplicate determinations. Similar results were received in at least two additional experiments.

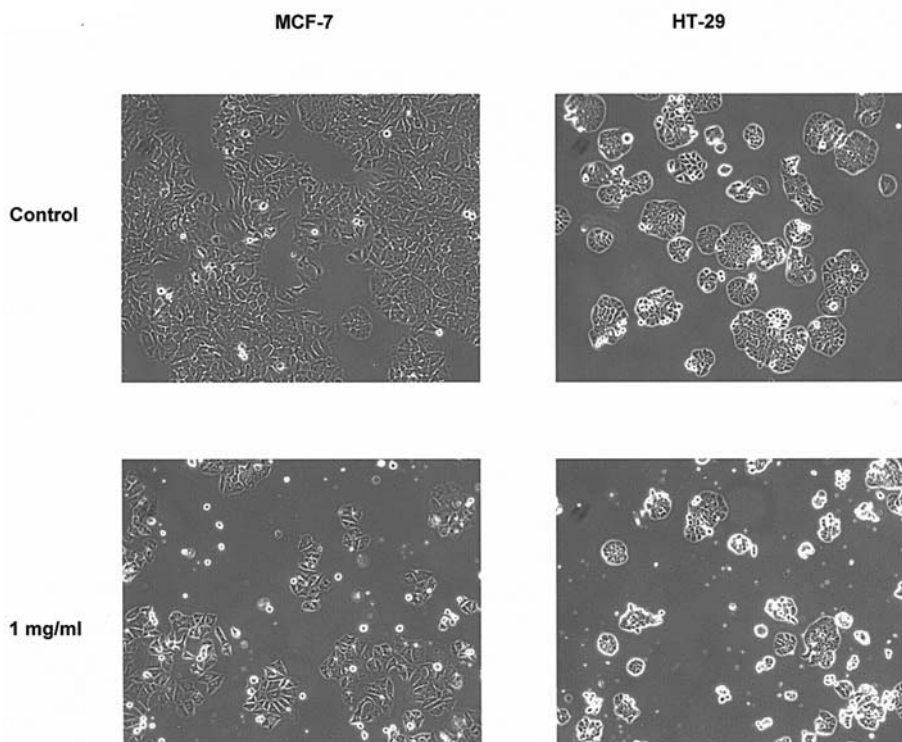


Figure 4. *P. ostreatus* induces morphological changes in cancer cells. MCF-7 and HT-29 cells were treated with *P. ostreatus* (0; 1.0 mg/ml) for 48 h and morphological changes evaluated as described in Materials and methods.

transcription factor 1 (E2F1), transcription factor Dp-1 (TFDP1) and proliferating cell nuclear antigen (PCNA) genes in breast cancer MCF-7 cells. Moreover, *P. ostreatus* induced p21, p19, p18, p15 and down-regulated CDK4, Ki-67, E2F1, TFDP1 and PCNA in colon cancer HT-29 cells (Table I).

Effects of P. ostreatus on cell cycle regulatory proteins. Cell cycle arrest at G0/G1 by *P. ostreatus* was recently reported in PC-3 cells (12). However, the molecular mechanism(s) responsible for the inhibition of growth of breast and colon cancer by *P. ostreatus* has not been previously addressed. To

confirm our DNA microarray data, whole cell lysates were prepared from MCF-7 and HT-29 cells treated with *P. ostreatus* (0-1 mg/ml) and subjected to Western blot analysis with p21, p53, PCNA, pRb and Rb antibodies. Thus, *P. ostreatus* significantly up-regulated expression of CDK inhibitor p21 and tumor suppressor p53 in MCF-7 cells (Fig. 6A). Moreover, we identified significant changes in the phosphorylation of retinoblastoma tumor suppressor protein Rb, whose expression and state phosphorylation play a pivotal role in the control of the G1/S cell cycle checkpoint (16). Expression of proliferating cell nuclear antigen

Table I. cDNA microarray analysis.

	MCF-7		HT-29	
	Gene	Fold change	Gene	Fold change
Up-regulated	p53	5.3	p21	2.1
	p27	1.3	p19	1.2
	p21	1.5	p18	1.5
	p19	1.3	p15	1.4
Down-regulated	CDK4	1.3	CDK4	1.2
	CDK6	2.2	TFDP1	1.5
	TFDP1	1.6	E2F1	5.6
	PCNA	1.2	PCNA	1.7
	Ki67	1.5	Ki67	2.0

cDNA microarray was performed and analyzed with Cell Cycle Oligo GEArray and GEArray Expression Analysis Suite Software as described in Materials and methods.

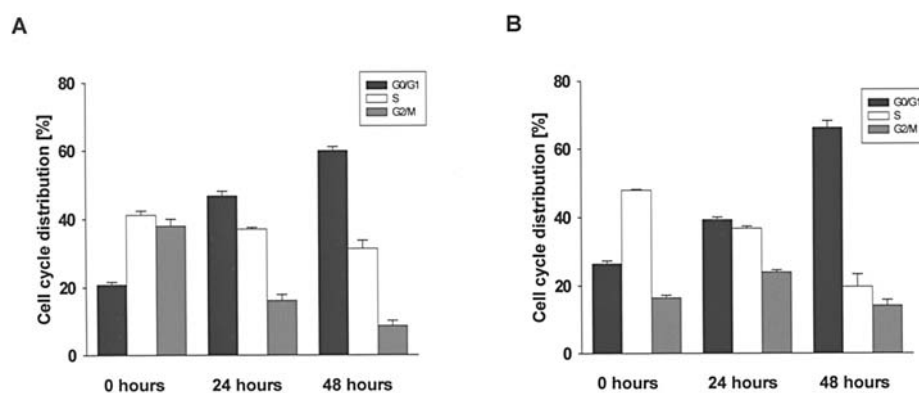


Figure 5. *P. ostreatus* induces cell cycle arrest at G0/G1 phase. (A) MCF-7, (B) HT-29 cells were treated with *P. ostreatus* (1.0 mg/ml) for 0, 24 and 48 h, and cell cycle distribution was evaluated by flow cytometry as described in Materials and methods. Data are the mean \pm SD of triplicate determinations.

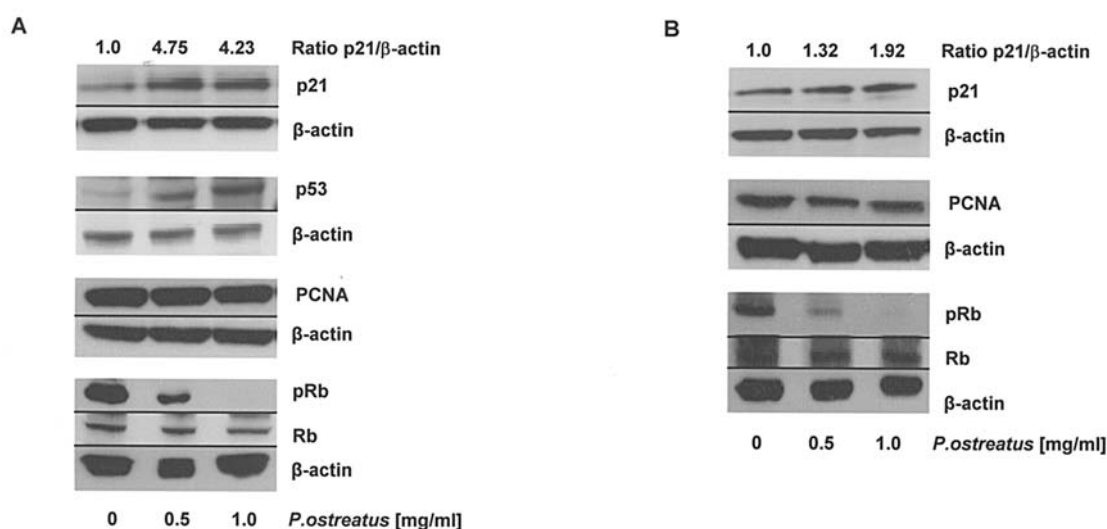


Figure 6. Effects of *P. ostreatus* on cell cycle regulatory proteins. (A) MCF-7 cells and (B) HT-29 cells were treated with *P. ostreatus* (0-1.0 mg/ml) for 24 h. The expression of p21, p53, PCNA, Rb and pRb was evaluated in whole cell extracts by Western blot analysis with the respective antibodies. The equivalent amount of protein was verified by reprobating the blot with anti- β -actin antibody. The results are representative of three separate experiments. The expression level of p21 (ratio p21/ β -actin) was quantified by densitometry as described in Materials and methods.

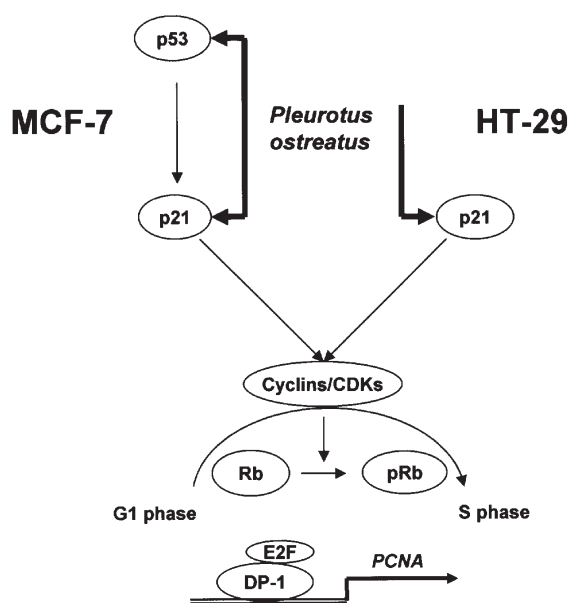


Figure 7. Schematic representation of the expression of cell cycle regulatory genes modulated by *P. ostreatus* in MCF-7 and HT-29 cells.

(PCNA), a protein involved in cell cycle regulation, DNA synthesis and DNA repair, was only slightly affected by *P. ostreatus* treatment in MCF-7 cells (Fig. 6A). Western blot analysis of HT-29 cell extracts treated with *P. ostreatus* revealed marked up-regulation of expression of CDK inhibitor p21. Moreover, *P. ostreatus* significantly inhibited phospho-Rb (pRb) with no effect on the levels of total Rb in HT-29 cells. However, expression of PCNA in HT-29 treated cells was not affected by *P. ostreatus* (Fig. 6B).

Discussion

The number of different mushroom species on earth is estimated at 140,000 of which maybe only 10% are known. Meanwhile, of those ~14,000 species currently recorded, ~50% are considered to possess varying degrees of edibility (17). Oyster mushrooms (*Pleurotus* species) are edible mushrooms and are widespread throughout the hardwood forests of the world (18). *Pleurotus ostreatus* is an edible mushroom widely researched for a variety of properties including antitumor effect. However, the molecular mechanism of its action on colon and breast cancer is not fully understood. Here, we demonstrated that *P. ostreatus* induces G0/G1 cell cycle arrest of MCF-7 cells by the up-regulation of the expression of p53 and p21, whereas cell cycle arrest of HT-29 is induced at G0/G1 by the up-regulation of the expression of p21.

Deregulated cell cycle progression is a common abnormality observed in human cancers. Progression through the cell-division cycle is regulated by the coordinated activities of cyclin/cyclin-dependent kinases (CDK) complexes. One level of regulation of these cyclin-CDK complexes is provided by their binding to CDK inhibitors (CKIs)(19). The CDK inhibitor p21 binds and inhibits the

cyclin D-, E-, and A-dependent kinases, regulating G₁ to S-phase transition of the cell cycle (20-22). Overexpression of p21 is reported to inhibit the proliferation of mammalian cells (21). In the present study, we observed by using cDNA microarray technology and Western blot analysis that *P. ostreatus* up-regulated expression of p21 and p53 in MCF-7 cells which have wild-type p53. Thus, treatment of MCF-7 cells by *P. ostreatus* may lead to the induction of p21 by p53 dependent mechanisms in MCF-7 cells. The increase of p53 protein levels in MCF-7 cells treated with *P. ostreatus* suggests that its growth inhibitory properties may involve the induction of DNA damage response. Moreover, we have found up-regulation of p21 in HT-29 cells at transcription levels and as well as on a protein level. As previously demonstrated, transcription of the p21 gene may be regulated by p53-dependent as well as p53-independent mechanisms (23). Our data indicated that *P. ostreatus* induces p21 expression in a dose-dependent manner in HT-29 cells, which lack functional p53, suggesting an independent mechanism. The p53 gene product is known to be a key player in the genotoxic-stress response in mammalian cells by inducing the transcription of p21, which in turn inhibits the cyclin E/CDK2-dependent phosphorylation of retinoblastoma (pRb)(24). Phosphorylation of Rb proteins disrupts binding to the E2F proteins, which allows them to act as transcription activators. E2F targets genes that are up-regulated as a result of Rb phosphorylation encode genes necessary for the completion of the G1/S phase transition, as well as genes necessary for DNA replication (25). In the present study, *P. ostreatus* dose dependently decreased the pRb level, indicating that up-regulation of p21 by *P. ostreatus* leads to inhibition of CDK activity, which results in decreased phosphorylation of CDK substrates. These results indicate that the decreased pRb contributes to the G1/S arrest observed in *P. ostreatus* treated cells. Moreover, cDNA microarray data showed decreased expression of E2F-1 and TFDP-1, members of the E2F transcription factor family in *P. ostreatus* treated MCF-7 and HT-29 cells, suggesting that the decrease in E2F-1 and TFDP-1 levels may also contribute to cell cycle arrest at the G0/G1 phase. In addition, p21 may also inhibit cell cycle progression via the interaction with proliferating cell nuclear antigen (PCNA) (26,27). PCNA protein is one of the molecules central in determining the life and death of the cell (28). The C terminus of p21, containing its nuclear localization signal, binds to and inhibits PCNA, thereby blocking DNA replication (29). The present study demonstrated that *P. ostreatus* slightly decreased the protein level of PCNA in MCF-7 treated cells, which may also contribute to the decreased DNA synthesis. However, expression of PCNA in HT-29 treated cells was not affected by *P. ostreatus*.

In conclusion, our data suggest that *P. ostreatus* significantly inhibits proliferation of human breast cancer cells MCF-7 and colon cancer cells HT-29 by G0/G1 cell cycle arrest. Therefore, *P. ostreatus* induces cell cycle arrest of MCF-7 by the up-regulation of the expression of p53 and p21, whereas cell cycle arrest of HT-29 is induced by the up-regulation of the expression of p21 (Fig. 7).

In summary, our data demonstrate that the dietary mushroom *P. ostreatus* specifically inhibits growth of colon

and breast cancer cells without significant effect on normal cells, and has a potential therapeutic/preventive effect on breast and colon cancer.

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