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Pleurotus eous polysaccharides suppress angiogenesis and induce apoptosis via ROS-dependent JNK activation and mitochondrial mediated mechanisms in MCF-7 human breast cancer cells

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Abstract

Breast cancer is one of the most prevalent cancers among women worldwide. Chemotherapy generally leads to drug resistance and severe side effects thus making it crucial to identify and develop highly efficient chemotherapeutic agents. Recently, edible mushrooms have been strongly investigated owing to their nutritional values and bioactive compounds with health benefits. The present study investigates the effects of polysaccharides isolated from the fruiting bodies of oyster mushroom, *Pleutorus eous* on MCF-7 human breast cancer cells. The viability of MCF-7 following exposure to *P. eous* polysaccharides (PEP) (50-250 µg/mL) were markedly decreased. A raise in the levels of Reactive Oxygen Species (ROS) and apoptotic cell counts were observed following PEP treatment. Futhermore, PEP down-regulated VEGF and Bcl-2 and raised caspase-3, caspase-9, Bax, phospho-JNK expressions and as well caused a significant decrease in mitochondrial membrane potential of MCF-7 cells. Thus, PEP effectively suppressed angiogenesis by down-regulating VEGF, and induced apoptosis.

Introduction

Breast cancer is one of the most prevailing cancers in women globally (Jemal et al., 2011; Noori and Hassan, 2012). The current treatment modalities, surgery, radiotherapy and chemotherapy are yet not effective (Bange et al., 2001) and also breast cancer remains highly resistant to chemotherapy (Hsu et al., 2005). Angiogenesis is vital for the growth and development of both primary and metastatic tumors (Rahman and Toi, 2003). Understanding angiogenesis thus represents a key factor in breast cancer development and metastasis (Schneider and Miller, 2005).

Currently, it has been considered that excessive production of reactive oxygen species (ROS) induces apoptosis, which could be exploited as an approach to kill cancer cells (Pan et al., 2009). Furthermore, ROS also induces various signaling pathways as mitogen-activated protein kinases (MAPK) signal transduction cascades (Pan et al., 2009). The c-Jun-N-terminal kinase (JNK), a member of MAPK family, has been reported to be vulnerable to ROS and plays a crucial role in mitochondrial dysfunction and subsequent initiation of apoptosis (Ip and Davis, 1998; Davis, 2000). Thus, targeted inhibition of appropriate signaling pathways, particularly ROS/JNK signaling, may possibly be effective in treatment and prevention of cancers.

Dietary supplements are used to overcome the toxic effects of chemotherapy or to increase the efficacy of therapy (Boon et al., 2000; Block et al., 2008). Edible mushrooms contain beneficial bioactive compounds and can be a good source for cancer treatment (Sarangi et al., 2006; Li et al., 2008; Stajic et al., 2009). Mushroom polysaccharides have been demonstrated to exhibit

direct inhibitory effects on cancer cell growth by modulating cell-cycle progression and inducing apoptosis (Wang et al., 2002).

Recent studies have demonstrated that polysaccharides from different oyster mushrooms - *Pleurotus sajor caju*, *Pleurotus florida*, and *P. abalones* inhibits cancer growth (Zhuang et al., 1993; Jose and Janardhanan, 2001; Wang et al., 2005; Li et al., 2012). In the present study, we investigated the effect of polysaccharides isolated from fruiting bodies of the oyster mushroom, *P. eous* on human breast cancer cells – MCF-7.

Materials and Methods

Antibodies and reagents

Dulbecco's modified eagle's medium (DMEM), RPMI medium 1640 and fetal bovine serum (FBS), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodiumditolyl-4,4-bis(2-azo-8-amino-1-naphthol -3,6-disulfonate) (trypan blue), 2,7-dichlorodihydrofluoresceindiacetate (DCFH-DA) and 5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethyl benzimidazolylcarbocyanine (JC-1) were purchased from Sigma (USA). Dimethyl sulfoxide (DMSO), sodium bicarbonate, penicillin-streptomycin, trypsin, polyvinylidenefluoride (PVDF) membrane and enhan-ced chemiluminescence (ECL) assay kit were purchased from Beyotime (China). The antibodies used in this study- Bcl-2, Bax, caspase-3 and caspase-9 (Oncogene, USA), Bax (Biomol, USA), VEGF, JNK, and phospho-JNK (Abcam, USA) and the HRP conjugated goat anti mouse/rabbit secondary antibodies (USA). All other chemicals were procured from Sigma-Aldrich.

Cell culture

Human breast carcinoma MCF-7 cells were obtained from American Type Culture Collection (ATCC). The cells were grown in RPMI-1640 medium supplemented with 10% FBS. The cell cultures were incubated in 95% room air and 5% CO_2 at 37°C, and passed thrice a week.

Isolation of P. eous polysaccharides

The dried fruiting bodies of *P. eous* were purchased from Gutian County, Fujian Province, China. The mushroom materials were thoroughly washed with tap water, air-dried and finely powdered. The crude polysaccharides were isolated as previously described (Yang et al., 2010). Briefly, the dried mushroom powder (200 g) was defatted with anhydrous ethanol. The mixture was filtered and the residues were collected, dried and extracted with hot water (1:10, w/v) at 80°C thrice for 60 min each time. The extracts were pooled and concentrated to 30% original volume under reduced pressure followed by centrifugation at 2,000 rpm for 15 min. The supernatant thus obtained was collected and three volumes of 95% alcohol was added slowly with constant stirring in order to precipitate the polysaccharides. The precipitate was incubated at 4°C overnight. Polysaccharide pellets were obtained by centrifugation at 4,000 rpm for 15 min and repeatedly washed sequentially with anhydrous ethanol, acetone and diethyl ether. The refined polysaccharide pellets were further completely dissolved in an appropriate volume of distilled water and intensively dialyzed for 48 hours against distilled water. The retentate portion was deproteinised by a freeze-thaw process (FD-1, Henan Yuhua Instrument Co., China), which was repeated 8 times, followed by filtration. Finally, the filtrate was lyophilised to yield crude polysaccharides (PEP).

Cell viability assay

To study the cell viability, MCF-7 cells (1×10^4 cells/well) were seeded in 96-well culture plates. After the cells reached 70% confluence, they were treated with various concentrations of PEP (50, 100, 150, 200 and 250 µg/mL) for 24 hours. At the end of incubation, cell proliferation was measured by MTT assay as described previously (Hordegen et al., 2006). Briefly, 10 µL of MTT stock solution (5 mg/mL) was added to each well and incubated for 4 hours at 37°C. The culture medium was then removed and 100 µL DMSO was added to dissolve the formazan crystals. After mixing, the absorbance was read at 570 nm with an ELISA reader (Bio-Rad, USA). Cell viability was expressed as the percentage of value against that of the solvent-treated control group.

Morphological observation of nuclear change

Morphological observation of nuclear change was assessed with Hoechst 33258 (Zhuo et al., 2009). MCF-7 cells (1×10^6 cells/mL) were seeded in 6-well plates and incubated with various concentrations of PEP for 48 hours at 37°C. Cells were then collected, washed, and fixed in 4% paraformaldehyde for 30 min and stained with 5 µg/mL Hoechst 33258 for 5 min at room temperature. The morphological changes were observed by visualizing the apoptotic cells using inverted fluorescence microscope (Nikon TE2000, Japan).

Detection of ROS generation

The generation of intracellular ROS was measured by flow cytometry using DCFH-DA staining. DCFH-DA is a non-fluorescent compound that can be enzymatically converted to highly fluorescent compound, DCF, in the presence of ROS. Cells were seeded at a density of 1×10^6 in 60 mm dishes, incubated for overnight and exposed to PEP (50-250 µg/mL) for 6 hours. Following treatment, cells were further incubated with DCFH-DA (10 µM) for 30 min at 37°C in dark. The cells were then washed twice with PBS and intensity of fluorescence was measured by flow cytometry (Lu et al., 2004).

Measurement of mitochondrial membrane potential (MMP)

Following exposure to PEP as in the determination of

ROS generation, the level of MMP was determined by flow cytometry using JC-1, a mitochondrial-specific cationic dye. JC-1 which is a monomer, emits green light (540 nm) when the membrane potential is lower than 120 mV, following excitation emits blue light (490 nm). At higher membrane potentials, JC-1 monomers convert to J-aggregates that emit a red light (590 nm) following excitation by green light (540 nm). Fluorescence was monitored at wavelengths of 490 nm (excitation)/540 nm (emission) and 540 nm (excitation)/590 nm (emission). Changes in the ratio between the measurement at wavelengths of 590 nm (red) and 540 nm (green) fluorescence intensities indicated the alternation of MMP level. Following treatment, cells were harvested, washed and incubated with JC-1 (25 µM) for 30 min at 37°C.

Western blotting analysis

For Western blotting analysis, 1 × 10^6 cells following exposure to PEP for 24 hours were collected and lysed in ice-cold RIPA buffer (50 mM Tris-HCl; 150 mM NaCl; 1 mM ethylene glycoltetraacetic acid (EGTA); 1 mM ethylenediamine tetraacetic acid (EDTA), 20 mM NaF,100 mM Na_3VO_4, 1% nonidet P-40 (NP-40), 1% Triton X-100, 1 mM phenylmethyl-sulfonyl fluoride (PMSF), 10 mg/mL aprotinin and 10 mg/mL leupeptin) for 30 min. Protein concentration was determined by Bradford method (Bradford, 1976). Cell lysates were electrophoresed on a 15% SDS polyacrylamide gel and transferred to PVDF membrane. Following blocking with 5% bovine serum albumin (BSA) in the mixture of tris-buffered saline and Tween-20 (TBST) for 60 min, the membranes were incubated with primary antibody overnight and followed by incubation with secondary antibody for 1 hour at room temperature. Protein bands were visualized using the ECL assay kit (Beyotime, Nantong, China). The band density was normalized to the expression of β-actin.

Statistical analysis

The data obtained were statistically analyzed using SPSS software (free trial version). The values are represented as mean ± SD, for three individual experiments. P values <0.05 are considered significant as determined by ANOVA (one-way analysis of variance).

Results

PEP at 100–500 µg significantly ($p<0.05$) decreased the cell viability (Figure 1). The viability was found to be 87.2% at 100 µg which gradually reduced with concentration and was 29.8% at 250 µg. The decrease in cell viability was nearly multi-fold at 250 µg.

To investigate the effect of PEP on nuclear morphology during cell apoptosis, we used Hoechst 33258. The staining showed considerable morphological changes in nuclear chromatin (Figure 2). The nuclei of untreated control MCF-7 cells were stained in less bright blue and homogeneous color. However following exposure to various concentrations of PEP, for 48 hours, most cells exhibited very intense staining of condensed and fragmented chromatin. The intensity of apoptotic cells increased gradually with the concentration of PEP with 250 µg exhibiting highest intensity. Only a few nuclei still displayed normal morphology on exposure to PEP.

ROS generation plays an important role in the pro-apoptotic activities (Simon et al., 2000; Thannickal and Fanburg, 2000). Generation of ROS upon PEP treatment

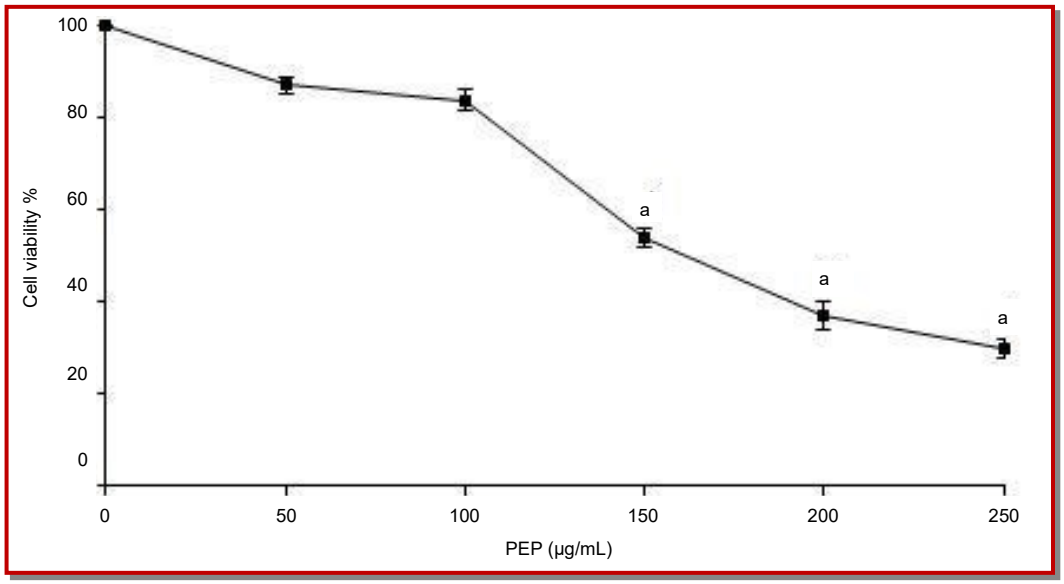

Figure 1: Effect of *P. eous* polysaccharides on the cell viability of MCF-7 cancer cells. Values are represented as mean ± SD; n = 3. [a]represents $p<0.05$ compared with control as determined by one way-ANOVA

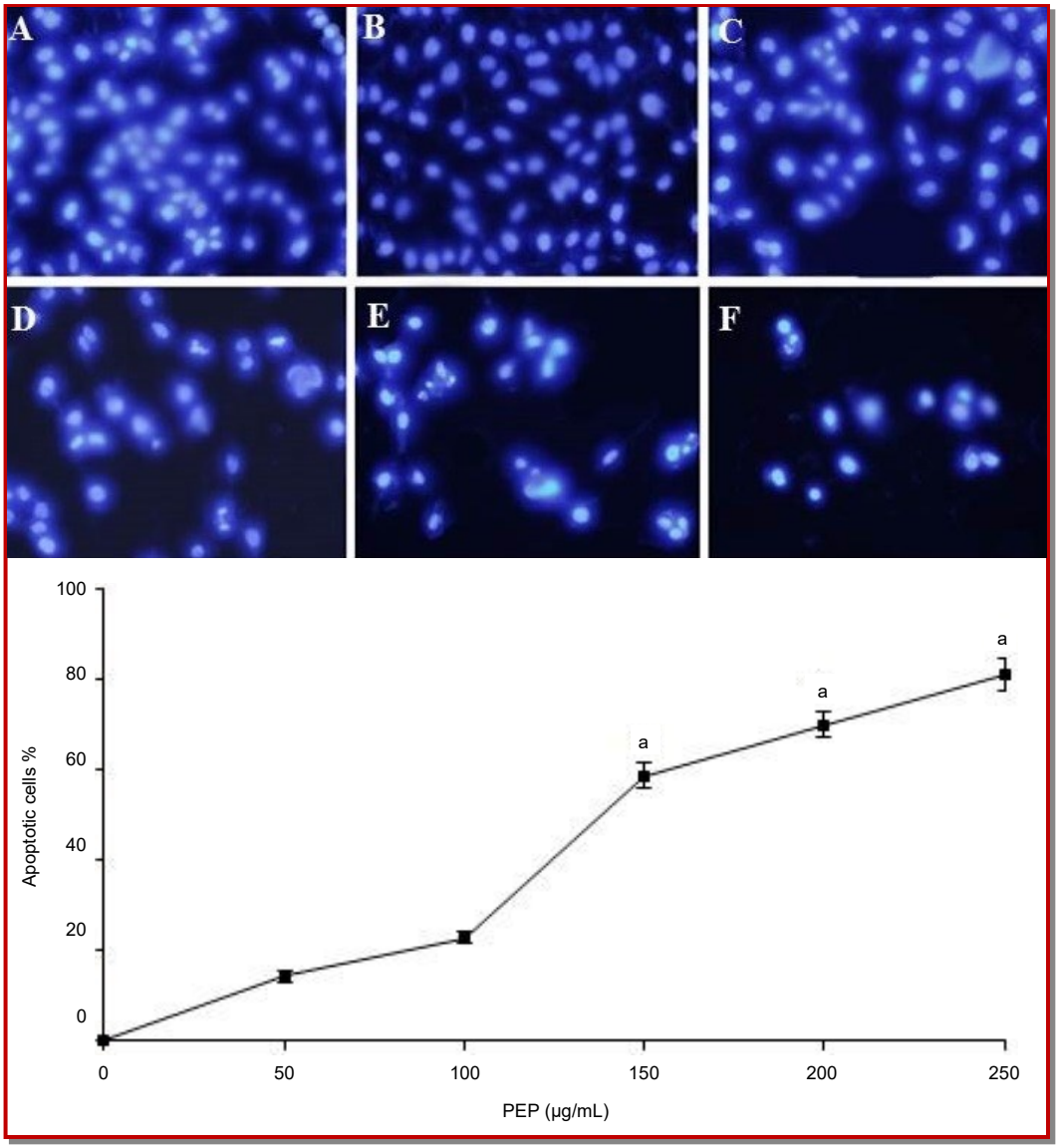

Figure 2: Influence of *P. eous* polysaccharides on apoptotic changes in the nucleus of MCF-7 cancer cells

Upper. Nuclear morphological changes in the MCF-7 cells (A represents control untreated cells, B-F represents MCF-7 cells exposed to 50–250 µg/mL of PEP respectively). Lower. Values are represented as mean ± SD; n=3; [a]represents $p<0.05$ compared with control as determined by one way-ANOVA

was measured by means of DCFH-DA and flow cytometry as an indicator of peroxides and superoxide accumulation. Upon exposure of MCF-7 cells to PEP at 50-250 µg, a concentration-dependent increase in ROS production was observed (Figure 3). Fluorescence intensities of PEP treated MCF-7 cells were significantly ($p<0.05$) higher than those of untreated controls.

Mitochondrial dependent pathway, one of the major molecular mechanisms of apoptotic induction (Estaquier et al., 2012), was also investigated in this study. Changes in mitochondria membrane potential (MMP) was evaluated by JC-1 staining. PEP treatment decreased MMP in MCF-7 cells in a dose-dependent manner (Figure 4).

Caspases are important regulators of apoptosis (Stennicke and Salvesen, 1998). In the PEP treated MCF-7 cells, there was a marked rise in the expressions of caspases-3 and 9 as compared to untreated MCF-7 cells. The increase in expression was observed to be dose-dependent (Figure 5). The expression of apoptosis-related proteins, particularly Bax and Bcl-2, in MCF-7 cells under the treatment of PEP for 24 hours evidenced a marked up-regulation in the expression of Bax but down-regulation of Bcl-2 in a dose-dependent manner. The most important regulator of human tumor angiogenesis is VEGF, also known as VEGF-A or

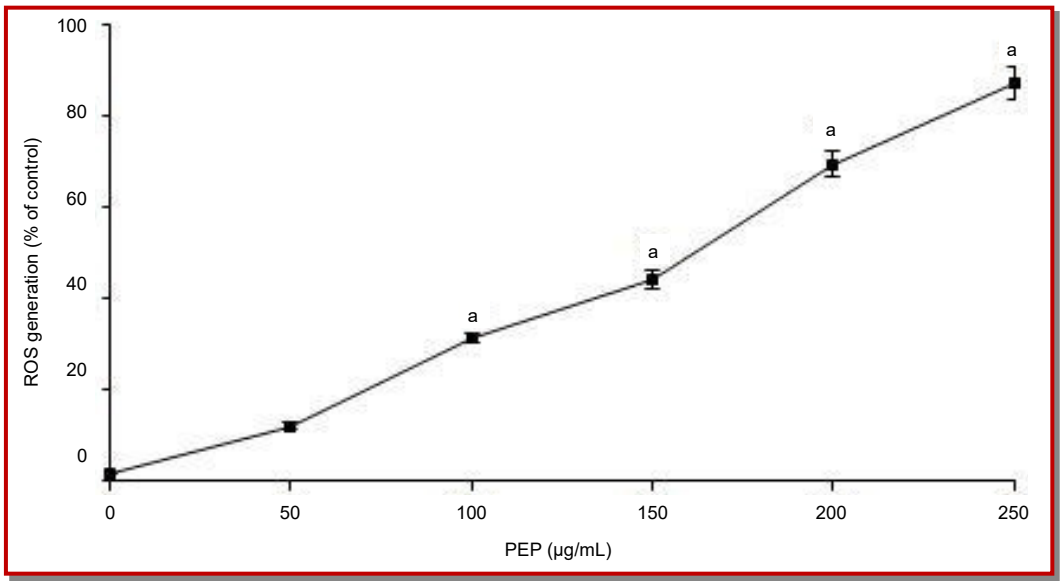

Figure 3: Influence of *P. eous* polysaccharides on intracellular ROS generation in MCF-7 cancer cells. Values are represented as mean ± SD; n=3. [a]represents $p<0.05$ compared with control as determined by one way-ANOVA

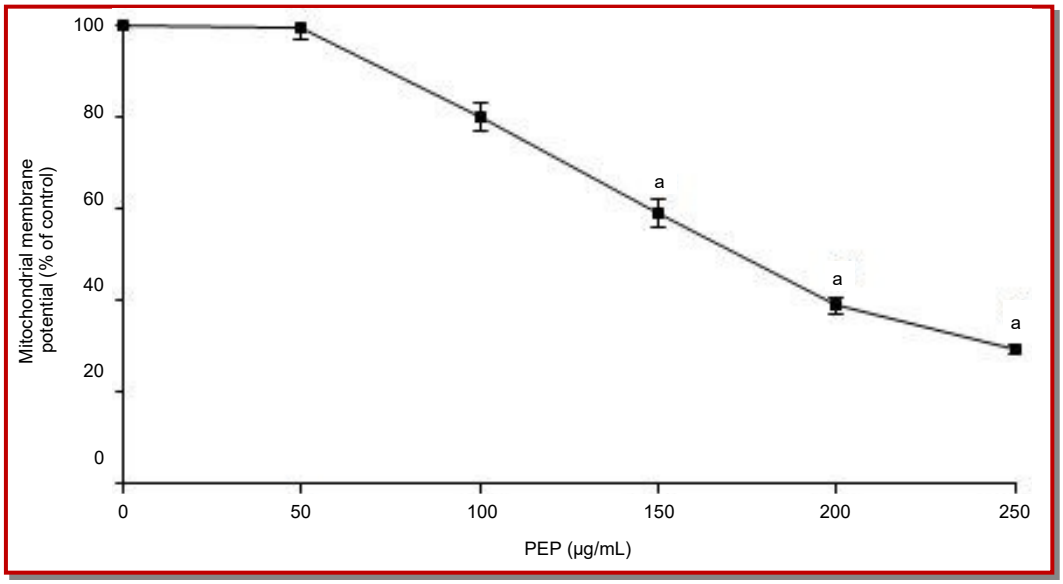

Figure 4: Effect of *P. eous* polysaccharides on the mitochondrial membrane potential of MCF-7 cells. Values are represented as mean ± SD; n=3. [a]represents $p<0.05$ compared with control as determined by one way-ANOVA

vascular permeability factor (Carmeliet, 2003). PEP at various concentrations (100-500 µg) markedly down-regulated VEGF expression in MCF-7 cells (Figure 6).

The c-Jun N-terminal kinase (JNK) pathway is one of the major signaling cassettes of the mitogen-activated protein kinase (MAPK) signaling pathway, which is also a key signaling modulator in cell apoptosis involved with ROS (Benhar et al., 2002). The probability of PEP exposure induced ROS generation to activate JNK signaling pathway was also assessed. The results showed that the expression of phosphorylated JNK was significantly increased with PEP treatment, while total JNK remained not much altered (Figure 6).

Discussion

Breast cancer is the most common malignant tumor in women. The therapeutic approaches include surgery and radiotherapy. However, both cause the severe pain and othe side effects (Siegel et al., 2012). Recent studies have aimed to identify novel agents that can inhibit proliferation of breast cancer cells with tolerable side effects. Medicinal or edible mushroom consumption is associated with the improvement of human health,

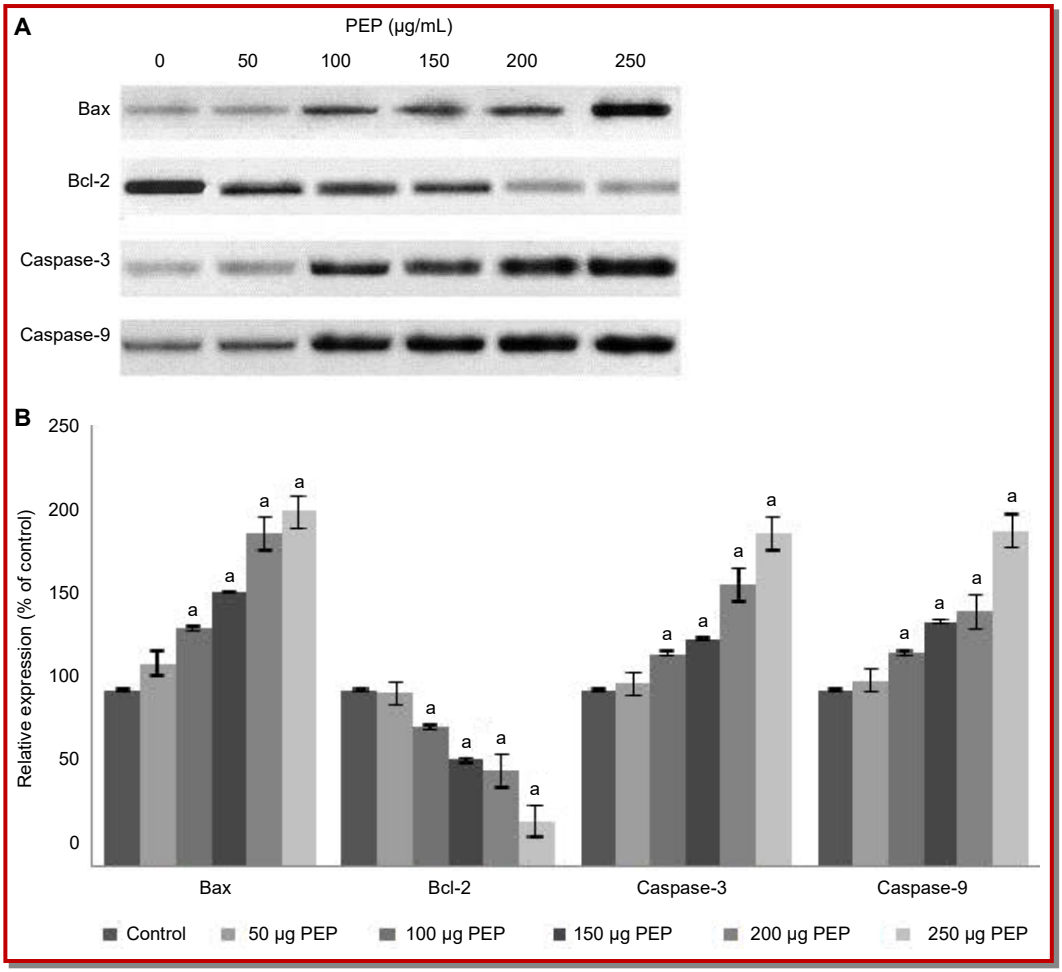

Figure 5: Effect of *P. eous* polysaccharides on the expression of apoptotic proteins

Relative expression of apoptotic proteins exposed to various concentrations of of *P. eous* polysaccharides. **B.** Values are represented as mean ± SD; n=3. [a]represents $p<0.05$ compared with control as determined by one way-ANOVA

especially for cancer prevention (Ko et al., 2005; Zhang et al., 2007; Li et al., 2012). Polysaccharides are the active constituents that are involved in these effects of mushrooms which can induce tumor cell death in several cancer types (Zhang et al., 2007; Wang et al., 2011). In the present study, we investigated the effect of polysaccharides isolated from fruiting bodies of pink oyster mushroom, *P. eous* in inducing apoptosis in MCF -7 cells.

PEP at various concentrations was able to markedly suppress cell growth and viability, suggesting its anti-proliferative activity. Earlier studies have indicated that polysaccharides derived from various mushrooms inhibit tumor growth (Lavi et al., 2006; Zhang et al., 2007; Song et al., 2011). Apoptosis is a highly synchronized death process by which cells undergo inducible non-necrotic cellular suicide. It plays a crucial role in anti-carcinogenesis (Kaufmann and Hengartner, 2001). Nuclear fragmentation, chromatin condensation and chromosomal DNA fragmentation are significant characteristic alterations that occur in apoptosis. Data obtained from the study evidence significant morphological changes in the MCF-7 cells exposed to PEP, thus indicating PEP-induced apoptosis in MCF-7 cells.

Studies have identified ROS as potential modulators of apoptosis by regulating both the extrinsic and intrinsic apoptosis pathways (Pelicano et al., 2004). ROS may possibly involve in the activation of death receptor primarily through inducing receptor clustering and formation of lipid-raft-derived signaling platforms (Circu and Aw, 2010). Oxidative stress due to ROS could stimulate an increase in metabolic activity and mitochondrial malfunction, resulting in the release of apoptogenic factors from mitochondrial inner membrane space and initiating apoptotic cascades. The results observed indicated that PEP induced ROS generation in a dose-dependent manner in MCF-7 cells, promoting apoptosis.

Increased levels of ROS are known to cause the depolarization of the mitochondrial membrane (Rogalska et al., 2008) which has been reported to be one of the

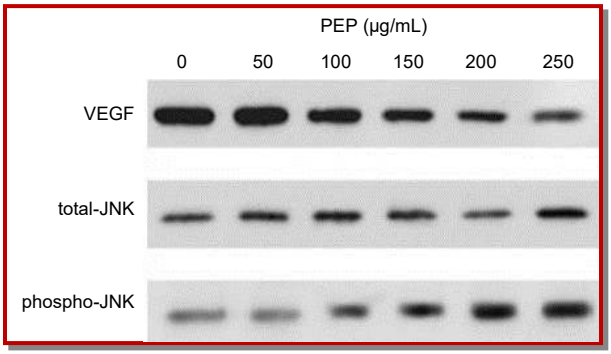

Figure 6: Influence of *P. eous* polysaccharides on VEGF and JNK expressions

earliest intracellular events of apoptosis (Desagher and Martinou, 2000; Han et al., 2006). In our study, exposure to PEP caused a marked decrease in MMP of PEP-treated MCF-7cells. It has been reported that ROS from mitochondria may oxidize membrane proteins of mitochondria and disturb the permeability of the outer membrane, leading to disruption of mitochondrial membrane potential, which could contribute to the release of cytochrome c and initiate apoptosis (Petrosillo et al., 2003). Thus, it could be suggested that increase in ROS induced by PEP could have been responsible for the disruption of MMP.

In line with the increased ROS levels, expression of caspases, caspase-3 and caspase-9 increased and indicate PEP induced apoptosis. Mitochondria mediated apoptosis is highly regulated by the Bcl-2 family proteins comprising both anti-apoptotic (Bcl-2, Bcl-xL) and proapoptotic members (Bax, Bak) and the balance between the expression levels of pro- and anti-apoptotic proteins is crucial for cell survival or cell death (Hengartner, 2000; Murthy et al., 2011). PEP treatment markedly enhanced Bax expression, and decreased Bcl-2 expression, suggesting that the change in the ratio of pro-apoptotic and anti-apoptotic Bcl-2 family proteins could contribute to the mitochondria-mediate apoptosis.

JNK, a member of the mitogen-activated protein kinases (MAPK) family, plays a vital role in cellular responses to a broad range of signals, as oxidative stressors. ROS has been reported as a potent activator of JNK by inhibiting the endogenous JNK inhibitors, such as JNK phosphatases (Zhang and Chen, 2004) thus sustaining JNK activation (Ray et al., 2012). Our results showed that PEP induced ROS generation and activated JNK as well. Activation of JNK could also cause the down-regulation of anti-apoptotic proteins such as Bcl-2 (Sinha et al., 2013). The results observed suggest that PEP-induced raised intracellular ROS, JNK activation plays a crucial role in eliciting early signals for triggering apoptosis.

Research data have shown that VEGF and its associated receptors are overexpressed in several types of human cancers, such as breast carcinomas (Yoshiji et al., 1996). An inverse correlation has been reported to exist between VEGF expression and overall survival in both node-positive and negative breast cancer (Gasparini et al., 1997, 1999). PEP was found to down-regulate VEGF expression indicating its effect on inhibition of angiogenesis.

Conclusion

The polysaccharides from *P. eous* inhibits cancer cell proliferation and induces apoptosis by increasing ROS generation, altering mitochondrial membrane potential, modulating apoptotic and anti-apoptotic protein expressions and also causing JNK activation. PEP inhibits angiogenesis by suppressing the expression of VEGF as well and thus stands as a potential candidate in cancer therapy.

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Conflict of Interest

Authors declare no conflict of interest

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