Anti-proliferative effect of Oyster mushroom, *Pleurotus* from Florida (misnomer) *P. florida* (Agaricomycetes) against HeLa and SIHA cervical cancer cells: Mushroom-boon for cancer therapies

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ABSTRACT

Introduction and Aim: Worldwide, people are suffering from the increased risk of cancer due to change in lifestyle, feeding habit and quality of food. To overcome this global problem, Mushroom act as a magic wand. The recent investigations reveal that cancer prevalence is inversely proportional to the intake of mushroom; this is because of its proteins, vitamins, carbohydrate and antioxidants contents.

Materials and Methods: Among various species of mushrooms, *Pleurotus* mushrooms are considered as nutraceuticals *i.e.* it has nutritional and medicinal values. However, mushroom products and extracts possess immunomodulating and direct cytotoxic effect on cancer cells.

Results: The results from the present study shows the potential cytotoxic effect of *Pleurotus* from Florida against cervical cancer (SIHA) cell line through apoptosis. When SIHA cells were incubated with varying concentration of methanolic extract of *Pleurotus* from Florida for time (48 hrs). The MTT assay revealed the cytotoxic activity of MME of *Pleurotus* from Florida in a dose dependent manner. The cell cycle analysis of the SIHA cells revealed that MME of *Pleurotus* from Florida have anti-cancerous activity.

Conclusion: The study could be concluded as the *Pleurotus* from Florida extract can be useful in the treatment of cervical cancers. The chemical compounds present in the *P.* from Florida might be useful in the development of drug for the treatment of cancer patients.

Keywords: Oyster mushroom; *Pleurotus;* methanolic extract; SIHA cells; HeLa cells; cytotoxic effect; propidium iodide; medicinal mushroom.

INTRODUCTION

Cancer is one of the most frightening diseases which take heavy toll of human lives. In developed countries such as USA cancer is the foremost health problems and recently out of four one person died with cancer in USA (1). Gynaecological cancer is chief cause of death in women worldwide (2). Cervical cancer is caused priory by infection with a high risk-group of human papilloma virus (HPV). The HPV oncogenic proteins like E6 and E7, play a crucial role in kicking off cervical cancer by interacting with p53and prb for the inactivation of these cell regulatory proteins sequentially. Immuno-stimulation by medicinal mushrooms usually occurs via innate immunity and is typically mediated by phagocytic cells (3).

These cells consume invading pathogens or interact with pathogen components, which in either case further stimulate innate and adaptive immunity through secretion of cytokines and chemokines (4). A major effort to reduce breast cancer mortality and morbidity is focused on developing better breast cancer prevention strategies. Hormonal blockade with tamoxifen has reduced the incidence of invasive and non-invasive breast cancer in high risk women. Moreover, *P. spp.* induced the expression of the tumour suppressor gene p53 and cyclin-dependent kinase (cdks) inhibitor p21 (CIP1/WAF1), whereas inhibited the phosphorylation of retinoblastoma Rb protein in MCF-7 cells (5). In addition, *P. spp.* also up-regulated expression of p21 and inhibited Rb-phosphorylation in HT-29 cells, suggesting that *P. spp.* suppresses the proliferation of breast and colon cancer cells via p53-dependent as well as p53-independent pathway**.**

Phenolic derivative compound Hispolon have capability to induce programmed cell death in breast and bladder cancer [**6]**. New discoveries in molecular oncology along with rapid expansion of our knowledge concerning the processes that govern differentiation, apoptosis, immune surveillance, angiogenesis, metastasis, cell cycle, and signal transduction control have revealed and abundance of specific molecular targets for cancer therapy, including a variety of small-molecule compounds that inhibit or stimulate these molecular targets. Medicinal attributes of *Pleurotus* traditionally is known as Medicinal Mushroom Mushrooms of *Pleurotus* species, commonly known as Oyster

mushroom are widespread throughout the hard wood forests of the world. They are known as an efficient blood pressure lowering agent, diuretic, cholesterol reducer, adjuvant and aphrodisiac and have also been shown to modulate the immune system (7). In addition, methanolic extract of *Pleurotus* from Florida (misnomer) *P. florida* exhibits anti-inflammatory and platelet aggregation inhibiting activities (8, 31). On the basis of dryness, the protein content of mushrooms may vary from 10 - 40% (9). Some amino acids like leucine, valine, glutamic acid, aspartic acid is present in huge quantity (10). All the essential amino acids are present in mushrooms while non-essential amino acid such as sulphur containing amino acid like cysteine and methionine are present in least quantity (11). Since antiquity mushroom used for their unique flavour and hedonistic food. Mushroom exerts legendary effects on promotion of health and it has prebiotic potential i.e. mushroom waste could reinforce the probiotic survival (12). Mushrooms have been used in Asian continent as traditional foods and medicines for a long time. There are various classes of primary and secondary metabolites in mushrooms that exhibit significant antimicrobial, antiviral and antitumor activities.

Wheat straw and paddy straw are used as substrates for cultivation of oyster mushroom and their derivatives are employed to lower the carcinogenicity in individuals. Various types of secondary metabolites like terpenes, flavonoids, phenol derivative compounds, steroids, lectins (carbohydrate + proteins) etc. are found in the oyster mushroom of which lectins primarily mitigate cancer (13, 27-30). It is ubiquitous in nature and show immune cell mediated activity against cancer (14, 20). It has low energy value due to least fatty acid component such as linoleic, palmitic acid, oleic acid (15, 19, 22). Mushroom consists of different types of macro & micro elements such as Mg, Ca, Cu, Fe, as well as many vitamin B complexes for instances niacin (B3), pantothenic acid (B5), riboflavin (B2) (16).

Mostly tribes residing near forests consume locally collected mushrooms which defeat various types of ailments and malnutrition related problems and provide appropriate dietary supplements. TSG, P53and cdk P21 inhibitors are induced by the extract of *Pleurotus* from Florida (17, 21, 23-26). In this study, we assessed the molecular mechanisms and inhibitory effects of ME using human cervical cancer cell lines (SIHA) cells. Our results showed that methanol extracts inhibited the growth of SIHA cells in dose-dependent manners. Furthermore, we found that ME showed significant antitumor effects by inducing G2/M phase arrest and apoptosis. These results are significant as they provide an insight into the molecular mechanism of ME which might be a potent chemotherapeutic agent for the treatment of cervical cancer. Ergosta-4,6,8(14),22-tetraen-3-one induces G2/M cell cycle arrest and apoptosis in human hepatocellular carcinoma HepG2 cells (18).

MATERIAL AND METHODS

Mushroom culture and cultivation

Culture of oyster mushroom (*Pleurotus* from Florida) was purchased from Directorate of Mushroom research (ICAR) Chambagaht (Solan)-H.P. 173213, India. Oyster mushroom culture maintained on malt extract agar medium at temperature 25 ± 2°C and pH 6-6.5 and sub-cultured at periodic interval of three week. Further, oyster mushroom was cultivated in our lab according to Singh *et al* 2016. In the present study, the oyster mushroom, *Pleurotus* from Florida was grown on paddy straw. The pure cultures of *P.* from Florida were procured from Directorate of Mushroom Research (DMR), Solan (HP), India and maintained on malt extract agar medium at temperature 25 ± 2°C and pH 6-6.5 and sub-cultured at periodic interval of three week.

Spawn preparation and Spawning

Spawn is referred as the vegetative mycelium of the fungus, which is grown on cereal grains. Wheat grains were washed and then half boiled. After that water from wheat grains was drained out. This was followed by mixing of $CaCO_3$ and $CaSO_4$ in 3:1 ratio. The wheat grains were now half filled in the bottles and plugged by cotton. The half-filled bottles were autoclaved at the temperature 121°C and pressure 15 psi for 30 minutes then left for overnight followed by inoculation of bottles with *P.* from Florida from cultured plate. Then bottles were incubated in BOD incubator at temperature 25 ± 2°C. After 3-4 days of inoculation fungal mycelium started spreading on the grains. The mycelium is white net web like in appearance. The bottles were nearly half filled in 10-12 days and in 18-21 days these were completely filled with white mycelial growth. Process of mixing spawn in the sterilized substrates is known as spawning. Treated paddy straw were mixed well and then 5% wet weight basis spawn grain was mixed to these substrates and filled to polythene bags. The mouth of the bags was tied with rubber band and holes were made to drain out extra water and for proper aeration.

Preparation of methanolic extracts

Fresh mushroom of fruit body dried in universal hot air oven (Thermolab India) at 500c until weight become constant. The mushrooms were grinding with mortar and pestle. Smashed biomass (100g) was suspended in 400 ml in absolute methanol and incubated (Metrex Orbital shaker Incubator) for 48 hr at 200 rpm at 370c. Suspension was filtered on Whatman No 1 (pore size 11µm) paper to remove biomass. This procedure repeated twice. Supernatant was put in Hot air oven at 500c until it dried. Finally, dried mass was dissolved in Millipore water to make 50mg/ml and stored at 40c.

Cell culture

The human cancer cell lines (HeLa and SIHA) were procured from National Centre of Cell Science (NCCS), Pune. Human Cervical Cancer Cell line (SIHA) were maintained in Dulbecco's modified Eagle's medium (DMEM) Containing gentamycin (50µ/ml) and 10% Foetal Bovine Serum (FBS). The culture was maintained at 370C in 5% CO_2 and 95% humidity.

Cell proliferation assay

The cytotoxic activity mushroom extract evaluated by the MTT {3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide} assay kit method according to the manufacturer's instructions (Cat- no. CCK003-1000, Himedia). HeLa and SIHA (cervical cancer) cell lines were cultured in a 96 well microtiter plate and treated at indicated time 48 hrs with various conc. (0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4mg/ml) of methanolic mushroom extracts of *P.* from Florida. After the completion of incubation period the observation was taken with a plate reader at 570 nm. The relative cell viability was calculated as the followed formula: Cell viability (%) = (OD treated/OD untreated) × 100%

Cell cycle analysis

HeLa cells primarily cultured and 3×10^6 were seeded in 6 well plate. After 48 hrs treated with *P.* from Florida extract (0.5, 1, 2, 3 mg/ml) for the 48 hrs. Cells were harvested by trypsinization, washed with Dulbecco's phosphate-buffer saline, and resuspended in Propidium Iodide (PI; Himedia cat-no. ML06) In brief cells were fixed with 70% chilled methanol and incubated for 3 hrs at room temperature. After incubation cells were resuspended in 250µg/ml RNase A {Merk} (500µl) for 2 hrs followed by staining with 10µg/ml PI solution and incubated for 15 minutes at room temperature. Cell cycle analysis was performed on a flow cytometer and analysed by cell quest software (BD, Becton Dickinson).

Statistical Analysis

The data were expressed mean, standard deviation and significance, standard error with mean.

RESULTS

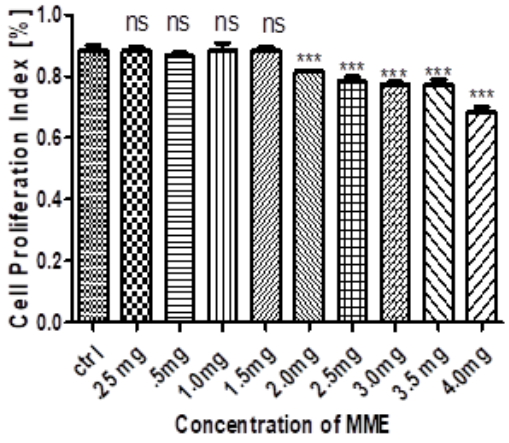

Figure. 1(a). Effects of MME on the proliferation of HeLa cells for 48 hrs *in vitro*.

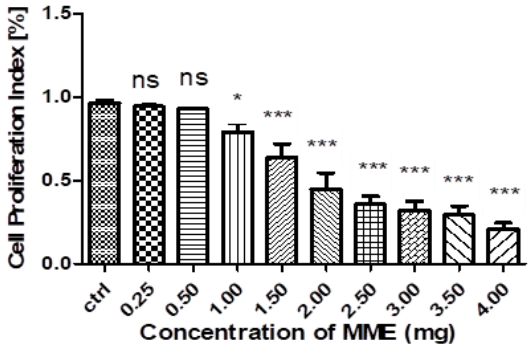

Figure. 1(b). Effects of MME on the proliferation of SIHA cells for 48 hrs in *vitro.* SIHA cells were treated for 48 hrs with various Conc. (mg/ml) of Methanolic mushroom extract and proliferation was assessed as described in Materials and methods. Data are the mean ± SD of triplicate determinations. Control; MME, Methanolic Mushroom Extract. One-way Anova $p<0.0001$

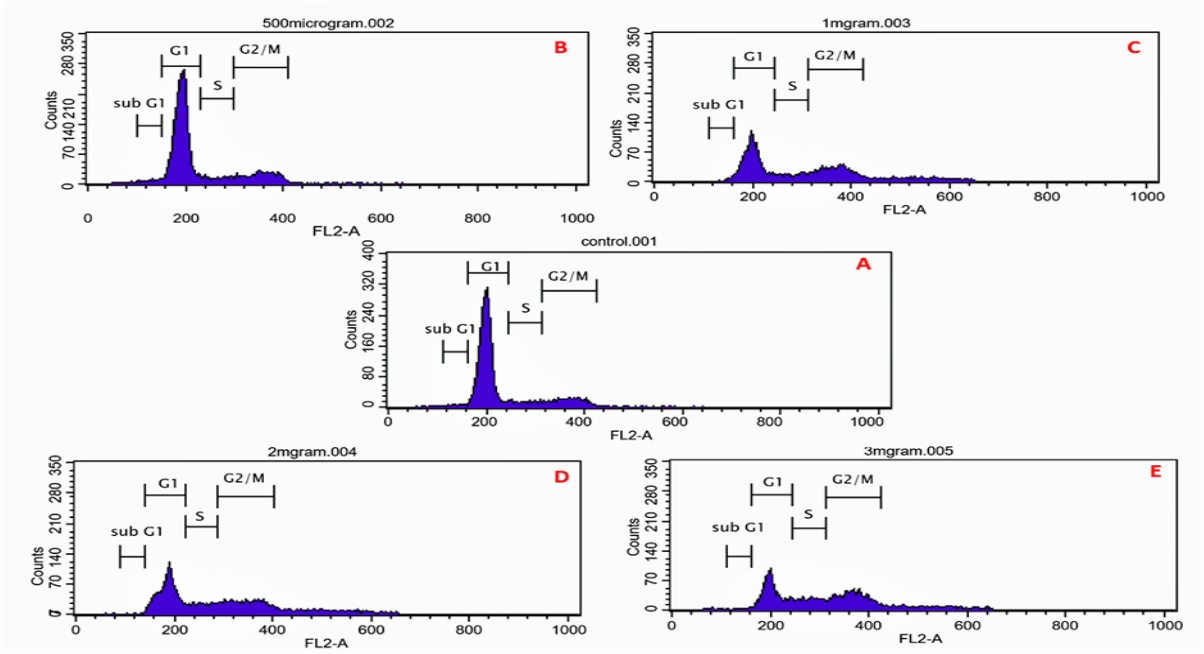

Figure. 2(a). Cell Cycle stage without MME, **(b)** Cell Cycle stage with 500µg MME Conc. **(c)** Cell Cycle stage with 1mg MME Conc. **(d)** Cell Cycle stage with 2 mg MME Conc. **(e)** Cell Cycle stage with 3 mg MME Conc.

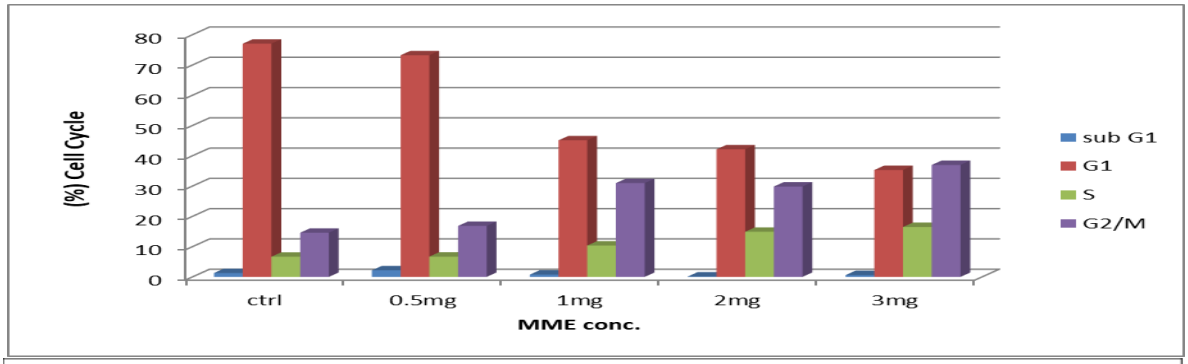

Figure. 3. Cell cycle distribution in SIHA cells upon MME treatment. SIHA cells were treated with different concentrations of MME for 48 hrs. After PI staining, cell cycle distribution was analysed by flow cytometry

DISCUSSION

MME of P. from Florida inhibits the multiplication of SIHA cervical cancer cell line and HeLa in vitro

To investigate the antitumor effect of P. from Florida, Hela and SIHA cells were exposed to ME of P. from Florida for 48 hrs at varying concentration and viability of cells was measured by MTT assay. P. from Florida extract showed significant cytotoxic effect on SIHA cells in dose dependent manner, but less significant changes were observed with Hela cells. From the statistical analysis in HeLa cells it is clear that in the beginning from 0.25mg to 1.5mg conc. no significance p value observed. In order to increase conc. from 2.0 mg to 4.0 mg then have more significance p value. In case of 1 mg conc. More standard deviation occurs. When SIHA cells are treated with same conc. and for the same time, in the beginning from 0.25mg to 0.5mg conc. no significance p value observed but in 1 mg have less significance p value observed. In order to increase conc. from 1.5 mg to 4.0 mg then have more significance p value. In case of 2 mg conc. more standard deviation occurs. So, the data suggest that SIHA cells are more susceptible to ME of P. from Florida in comparison to Hela cells.

MME of P. from Florida induces cell cycle arrest in SIHA cells

Further, in order to detect the affected cell cycle due to P. from Florida, SIHA cells were treated with different concentrations of ME of P. from Florida for 48 hr and stained with PI and analysed by flow cytometry. After ME of P. from Florida treatment, a dose dependent accumulation of SIHA cells at G2/M-phase was observed after 48 hr of exposure and its frequencies increased from 15% (untreated) to 37% in dose dependent manner. So these results suggest that ME of P. from Florida induces the G2/M cell cycle arrest in SIHA cells. From above it is concluded that mushroom extracts possess anti-cancerous activity.

CONCLUSION

In summary, it is clear from aforementioned result that in modern time mushrooms can play highly significant

roles in human life in the form of dietary materials as well as in the form of sources for production of various pharmaceutical materials. MME possess anti-cancerous attributes in cervical cancer cell line especially in SIHA. Furthermore, it is also clear that mushrooms have promising application in cancer treatment. There is need of more brawny research in the field of mushroom for its better utilization in cancer treatment as well as in cure of other ailments. MME inhibited SIHA cell growth through induction of arresting of cell cycle in G2/M stage, 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. However, the components of ME and its antitumor mechanism need to be further investigated sand future study, we will prolong the drug administration to detect the effect of P. from Florida on the survival of tumour mice.

In conclusion, our data suggest that P. from Florida significantly inhibits proliferation of human cervical cancer cells SIHA by G2/M cell cycle arrest. In summary, our data demonstrate that the dietary mushroom P. from Florida specifically inhibits growth of cervical cancer cells without significant effect on normal cells and has a potential therapeutic preventive effect on cervical cancer.

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REFERENCES

1. Jedinak, A., Sliva, D. *Pleurotus ostreatus* inhibits proliferation of human breast and colon cancer cells through p53-dependent as well as p53-independent pathway. Int J Oncol. 2008; 33: 1307-1313.
2. Sliva, D., Sedlak, M., Slivova, V., Valachovicova, T., Lloyd, F. P., Jr, Ho, N. W. Biologic activity of spores and dried powder from *Ganoderma lucidum* for the inhibition of highly invasive human breast and prostate cancer cells. J Altern Complement Med. 2008; 9: 491-497.
3. Moharib, S. A., Maksoud, El., Abd, N., Halla, M., Mahmoud, R., Shehata, M. Anticancer activities of mushroom polysaccharides on chemically induced colorectal cancer in rats. J App Pharm Sci. 2014; 4: 054-063
4. Lindequist, U., Niedermeyer, T. H., Jülich, W. D. The Pharmacological Potential of Mushroom. Evid Based Complement Alternat Med. 2005; 2: 285-299.
5. Chen, Y. J., Shiao, M. S., Lee, S. S., Wang, S. Y. Effect of *Cordyceps sinensis* on the proliferation and differentiation of human leukemic U937 cells. Life Sci. 1997; 60: 2349-2359.
6. Cheung, L. M., Cheung, P. C., Ooi, V. E. Antioxidant activity and total phenolics of edible mushroom extracts. Food Chemistry. 2003; 381: 249-255.
7. Jose, N., Ajith, T. A., Janardhanan, K. K. Antioxidant, Anti-inflammatory, and Antitumor Activities of Culinary-Medicinal Mushroom *Pleurotus pufmonanus* (Fr.) Quel. (Agaricomycetideae). Int J Med Mushr. 2002; 4.
8. Jose, N., Ajith, T. A., Janardhanan, K. K. Methanol extract of the oyster mushroom, *Pleurotus florida*, inhibits inflammation and platelet aggregation. Phytother Res. 2004; 18: 43-46.
9. Choi, D., Cha, W. S., Kang, S. H., Lee, B. R. Effect of *Pleurotus ferulae* extracts on viability of human lung cancer and cervical cancer cell lines. Biotechnology and Bioprocess Engineering. 2004; 9: 356-361.
10. Grube, B. J., Eng, E. T., Kao, Y. C., Kwon, A., Chen, S. White button mushroom phytochemicals inhibit aromatase activity and breast cancer cell proliferation. J Nutr. 2001; 131: 3288-3293.
11. Chang, S. T., Miles, P. G. Edible mushroom and their cultivation. Book: Edible mushrooms and their cultivation. 1989; pp,345 pp, ISBN : 084936758X, Record Number : 19900395757, Publisher : CRC Press, Location of publication : Boca Raton Country of publication : USA.
12. Chou, W. T., Sheih, I. C., Fang, T. J. The Applications of Polysaccharides from Various Mushroom Wastes as Prebiotics in Different Systems. Journal of Food Science. 2013; 78(7): M1041-M1048.
13. Adachi, Y., Ishii, T., Ikeda, Y., Hoshino, A., Tamura, H., Aketagawa, J., Ohno, N. Characterization of Glucan Recognition Site on C-Type Lectin, Dectin 1. Infection and Immunity. 2004; 72(7): 4159-4171.
14. Baars, J. J. P., Hendrickx, P. M., Sonnenberg, A. S. M. Prototype of a sporeless oyster mushroom, Mushroom Sci. 2004; 16: 139-147.
15. Brodie, A., Lu, Q., Nakamura, J. Aromatase in the normal breast and breast cancer. J. Steroid Biochem. Mol. Biol. 1997; 61: 281-286.
16. Chen, S., Sun, X. Z., Kao, Y. C., Kwon, A., Zhou, D., Eng, E. Suppression of breast cancer cell growth with grape juice. Pharm. Biol. 1997; 36: 53-61.
17. Chen, Y. J., Shiao, M. S., Lee, S. S., Wang, S. Y. Effect of *Cordyceps sinensis* on the proliferation and differentiation of human leukemic U937 cells. Life Sci. 1997; 60: 2349–2359
18. Zhao, Y. Y., Shen, X., Chao, X., Ho, C. C., Cheng, X. L., Zhang, Y., Sun, W. J. Ergosta-4,6, 8(14), 22-tetraen-3-one induces G2/M cell cycle arrest and apoptosis in human hepatocellular carcinoma HepG2 cells. Biochimica et Biophysica Acta (BBA). General Subjects. 2011; 1810(4): 384-390.
19. Gao, Y., Zhou, S. Cancer prevention and treatment by *Ganoderma*, a mushroom with medicinal properties. Food RevInt. 2003; 19: 275-325.
20. Gao, Y., Zhou, S. The immunomodulating effects of *Ganoderma lucidum.* Int J Med Mushrooms. 2002; 4: 1-17.
21. Allgayer, H. Molecular regulation of an invasion-related molecule - options for tumour staging and clinical strategies. Eur J Cancer. 2006; 42: 811-819.
22. Hajjaj, H., Mace, C., Roberts, M., Niederberger, P., Fay, L. B. Effect of 26-oxygenosterols from *Ganoderma lucidum* and their activity as cholesterol synthesis inhibitors. Appl. Environ. Microbiol. 2005; 71: 3653-3658.
23. Hetland, G., Johnson, E., Lyberg, T., Bernardshaw, S., Tryggestad, A. M. A. Grinde, B. Effects of the Medicinal Mushroom *Agaricus blazei Murill* on Immunity, Infection and Cancer, Article in Scandinavian Journal of Immunology. 2008; 1365-3083.
24. Hetland, G., Sandven, P. β-1,3-glucan reduces growth of *Mycobacterium bovis* in macrophage cultures. FEMS Immunol Med Microbiol. 2002; 33: 41-5.
25. Hsieh, T. C., Wu, J. M. 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. 2001; 18: 81-88.
26. Hsieh, T. C., Kunicki, J., Darzynkiewicz, Z., Wu, J. M. Effects of extracts of *Coriolus versicolor* (I'm-Yunity) on cell-cycle progression and expression of interleukins-1 beta, -6, and -8 in promyelocytic HL-60 leukemic cells and mitogenically stimulated and non-stimulated human lymphocytes. J Altern Complement Med. 2002a; 8: 591-602.
27. Huang, B. M., Chuang, Y. M., Chen, C. F., Leu, S. F. Effects of extracted *Cordyceps sinensis* on steroid genesis in MA-10

mouse Leydig tumor cells. Biol. Pharm. Bull. 2000; 23: 1532-1535.
28. Liu, X., Zhou, B., Lin, R., Jia, L., Deng, P., Fan, K., *et al.* Extraction and antioxidant activities of intracellular polysaccharide from *Pleurotus* sp.. mycelium. Int. J. Biol. Macromol. 2010; 47: 116-119.
29. Oshiman, K., Fujimiya, Y., Ebina, T. *et al.* Orally administered beta-1,6-d-polyglucose extracted from Agaricus blazie results in tumour regression in tumour-bearing mice. Planta Med. 2002; 68: 610-614.
30. Prasad, S., Rathore, H., Sharma, S., Yadav, A. S. Medicinal Mushrooms as a Source of Novel Functional Food, International Journal of Food Science, Nutrition and Dietetics (IJFS) 2015; ISSN 2326-3350.