



Effectiveness of *Pleurotus ostreatus* Extract Through Cytotoxic Test and Apoptosis Mechanism of Cervical Cancer Cells

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Abstract

Pleurotus ostreatus is a common mushroom cultivated in Indonesia, with potential properties of bioactive compounds for medicinal mushroom. This study was aimed at obtaining *P. ostreatus* extract bioactive compounds potential in inhibiting the proliferation of cervical cancer cells (HeLa) and evaluating the HeLa cell proliferation kinetics and HeLa cell death mechanisms. The research was beneficial in making this product can be easily applied in a more controlled industrial scale. Anticancer activity test through a cytotoxic test using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], the kinetics proliferation of HeLa cells and HeLa cell death mechanism were performed. Linear regression analysis was used to analyze the data. Ethyl acetate extract of *P. ostreatus* isolated from Madiun showed the best results with $IC_{50} = 107.59 \mu\text{g} / \text{ml}$. HeLa cell proliferation kinetics analysis showed that the application of bioactive compounds $100 \mu\text{g} / \text{ml}$ resulted in an increase of in death of HeLa cells along with length of incubation time. An important finding was that HeLa cells death by apoptosis was greater than by necrosis. In conclusion, the extracts of *P. ostreatus* has the potential to inhibit the growth of HeLa cells.

How to Cite

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INTRODUCTION

Pleurotus ostreatus (Jacq.) P. Kumm. is one type of edible mushroom that is cultivated in the several regions in Indonesia. In Indonesia, *P. ostreatus* is known as the oyster mushroom, this is because the attribute of this mushroom of clean white color and shaped like oyster with the attachment of the stalk which is not exactly in the middle of the hood. According to Stamets (2000) white oyster mushroom has a hood (pileus), stem (stipe) and lamella (lamellae).

Bioactive compounds of *P. ostreatus* (oyster mushroom) cultivated in subtropical countries has been examined of its benefit to health. However, studies on anticancer from bioactive compound of *P. ostreatus* developed in Java has never been done before. Information on the potential of bioactive compounds of *P. ostreatus* has been stated by Poucheret et al. (2006) that *P. ostreatus* can be used as a medicine and has been widely studied in several countries, including Japan, China, Korea, and Brazil. The search of anticancer compound is still continuing as there are few safe cancer drugs, especially those with no interference to the healthy cells.

Cancer is one of the many types of diseases that cause death and may occur in human regardless of age group, gender or race. Data from the Ministry of Health reported that about 6% or 13.2 million of Indonesian people are suffering from cancer and this disease is the fifth leading cause of death in Indonesia. Cancer of the cervix (cervical) is the most common cancer in women that cause death. In addition to breast cancer, cervical cancer is the second most common worldwide cancer in women over the age of 15 years. The main cause of this cancer is due to Human Papilloma Virus (HPV). In the world, every two minutes a woman dies of cervical cancer. In Indonesia, this type of cancer ranked first by frequency of occurrence (Tjindarbuni & Cipito, 2002).

Cancer therapeutic strategies generally combine surgery with chemotherapy and radiation, but until now there is no standardized therapy is right for cancer. The negative side effect of cancer therapy strategies encourages intensive research and development of cancer drugs. One approach that is now getting attention is the use of non-toxic chemoprevention compound combined with chemotherapeutic agents to increase its efficacy by reducing toxicity to normal tissue. According to Lindequist et al. (2005); Poucheret et al. (2006); Gregory et al. (2007) & Kai (2007), *P. ostreatus* is not only an edible mushroom, but

also capable of producing bioactive compounds that have the potential as a drug (*medicinal mushroom*). Chakravarty (2011) also states that the oyster mushroom can produce polysaccharides (β glucan), which has immunomodulatory and anticancer activity. Dewick (2002) and Kai (2007), states that macroscopic fungi potentially produce bioactive compounds such as plants, including alkaloids, terpenoids, steroids, flavonoids, peptides, polysaccharides, lignans and others. Research by Manjunathan and Kaviyaran (2011) showed that different carbon sources, nitrogen and minerals greatly affect the antimicrobial activity of isolates of *L. tuberregium*.

P. ostreatus has several benefits, including anticancer, antioxidant, lowering blood pressure and cholesterol levels (Wasser, 2002; Lindequist et al., 2005; Fan et al., 2006). Some bioactive compounds that have been isolated both intracellular and extracellular are pleuran (β -glucan) and terpenoids. Pleuran (β -glucans) as the main compound produced by *P. ostreatus* is a group of polysaccharides (Smith et al., 2002; Hozova, 2004).

Research on the development of drug from fungus was based on the use of fungal fruit bodies, mycelium and its culture filtrate obtained through fermentation techniques. The production of bioactive compounds from *P. ostreatus* was based on the cultivation either in liquid or solid media. Cultivation in liquid medium through fermentation, will be yielding mycelium and culture filtrate of which metabolites extract obtained. While cultivation on a solid medium proceeded by planting the mushrooms on sawdust medium which is commonly known as mushroom cultivation, may produce extractable metabolites from the fruit body (Ekowati et al., 2009). Extraction is one of the methods to obtain bioactive compounds. And the choice of extraction method depends on the nature of materials and compounds to be isolated. It is suspected that the differences of solvent polarity may produce extract of bioactive compounds with different anticancer potential.

There is very limited research on the effectiveness of bioactive compounds from the culture filtrate and the fruit body of *P. ostreatus* for anticancer purpose. While the information regarding the source of the extract and their effective concentration to inhibit proliferation of the cervical cancer cells is very important. *P. ostreatus* originating from several different cultivation locations is thought to have different abilities to produce bioactive compounds as the biosynthetic processes is either influenced by genes or affected by physical and chemical environment factors. Ex-

red by *ELISA reader* at a wavelength of 550 nm. Results of absorbance reading were converted into percentages life of HeLa cells to obtained IC₅₀ values of the examined compounds. Observation on cell proliferation kinetics toward the addition of examined solution was conducted as that of diluting compound extract concentration incubated 0 hour, 24 hours, 48 hours and 72 hours.

DNA staining with acridine orange/ethidium bromide was carried out as follows : Cover slips were put into a 24-well plate and the cells were distributed on them, incubated for 24 hours in a CO₂ incubator 5%, then added with test solution with concentrations determined from the previous treatment, and incubated for 24 hours. At the end of incubation, RPMI media was taken, then the cover slips were lifted from the wells and placed on an object glass, drip with acridine orange/ethidium bromide. Observations mechanisms of cell death is done with a fluorescence microscope. Observation of cell death mechanism of apoptosis and necrosis quantitatively was done by calculating the percentage of living and dead cells, either through the mechanism of apoptosis and necrosis using Annexin V-FITC Apoptosis Detection Kit by means of flow cytometry.

The data obtained for the cytotoxic activity was analyzed using linear regression. Analysis of the mechanism of death (mortality percentage in apoptosis and necrosis) is done using the method of Flow Cytometry. The morphology of cell death were analyzed descriptively.

Data of cytotoxicity assay (absorbance) is converted to life percentage of cell with the following formula:

$$\text{Life percentage of cell} = \frac{\text{absorbance of test wells} - \text{absorbance media controls}}{\text{absorbance of control cells} - \text{absorbance media controls}} \times 100\%$$

Life percentage of cell data were converted to the IC₅₀ (Inhibition Concentration 50) using the regression analysis

RESULTS AND DISCUSSION

Bioactive compounds was derived from the extract of fruit bodies and culture filtrate fermentation of *P. ostreatus*. The preliminary research showed that within 28 days of incubation, *P. ostreatus* produced optimal bioactive compounds, due to the growth of the fungus has entered the stationary phase, there was no more growth. Extraction of bioactive compounds was performed using chloroform and ethyl acetate as a non-polar and semi-polar organic solvents respectively, resulting two different compounds extract.

Cytotoxic test performed with MTT method to quantify HeLa cell growth inhibition by the extract of *P. ostreatus*. This assay was to determine the concentration of the examined sample that can inhibit the growth of HeLa cells by 50% (IC₅₀). The results of cytotoxic bioactive compound assay of *P. ostreatus* shown in Table 1.

Analysis to determine the value of IC₅₀ on HeLa cells showed that bioactive metabolites from *P. ostreatus* inhibited the growth of HeLa cells with IC₅₀ values varied (Table 1). The lowest IC₅₀ value of the ethyl acetate extract was 107.59 ug/ml. The smaller the IC₅₀ values indicated the more active extracts to kill HeLa cells. *P.ostreatus* isolated from Madiun resulted in the lowest IC₅₀. Fungal isolates originating from different locations have genetic variation, as reported by Ekowati *et al.* (2012) that there are genetic variations and kindship with the genetic distance between 2-58% within eight strains of *P.ostreatus* in Java. There are researches using the same method which has been reported. Cytotoxic test by Israilides *et al.* (2008) using the MCF-7 cancer cells showed that IC₅₀ of the fruit body

Table 1. IC₅₀ value of bioactive compounds from the extracts of *P. ostreatus*

Treatment	IC ₅₀ (ug/ml)
Ethyl acetate extract of the fruit bodies Po Md	107.59
Ethyl acetate extract of the fruit bodies Po MI	136.21
Ethyl acetate extract of the fruit bodies Po P	266.81
Ethyl acetate extract of the filtrate Po Md	456.34
Chloroform extract of the fruit body Po Md	140.45
Chloroform extract of the fruit body Po MI	249.94
Chloroform extract of the fruit body Po P	218.87
Chloroform extract of the filtrate Po Md	477.68

Description: Po Md (*P. ostreatus* from Madiun), Po MI (*P. ostreatus* from Malang), Po P (*P. ostreatus* from Purwokerto)

died at 72 hours of incubation time. This gave a clear illustration that the bioactive compounds of *P. ostreatus* potentially inhibit the proliferation of HeLa cells, as the percentage of living HeLa cells was strongly influenced by the length of exposure to the extract.

Morphology of live and dead HeLa cells, can be seen through the observation and by staining the cells with acridin orange-ethidium bromide (double staining). Dead cell due to apoptosis mechanism appears to form spheres and will separate into apoptotic bodies and then be destroyed by macrophages. According to Brady (2007), apoptosis is a programmed cell death, so it does not cause an inflammatory reaction, whereas necrotic cell death can cause inflammatory reactions in the body. Apoptosis is a process of cellular metabolism that results in the destruction of cells with specific cell morphology. Cells that will die from apoptosis, forms a bulge in the cell membrane (blebbing), cytoplasmic volume shrinkage, chromatin condensation and DNA fragmented into smaller size, and appear to form the bodies of apoptosis (apoptotic body). Meiyanto et al. (2008) suggested that in the acridin orange-ethidium bromide staining, dead cells fluorescent orange, whereas the living cells fluorescent green. The orange fluorescence of dead cells indicate the loss of a membrane permeability in some cells due to exposure to the extract causing ethidium bromide can enter the cell and orange fluorescence happens as an indicator of cell death.

Methods of cell staining with ethidium bromide acridin-orange (double staining) are less effective to distinguish between cell death through the mechanism of apoptosis and necrosis. Using such methods, it is very difficult to know the number of dead cells through those mechanism, so examination necessarily continued with apoptosis test using flow cytometry. The percentage of cells that die by apoptosis and necrosis was shown in Figure 4.

Observation of apoptosis and necrosis in HeLa cells by flowcytometry showed that cells that were exposed to 100 ug/ml ethyl acetate extract of the fruit body PoMd for 24 hours was able to kill HeLa cells up to 59.34%. HeLa cell death caused by apoptosis mechanism was 34.97% and 24.37% by necrosis. This suggests the capacity of the bioactive compounds from *P. ostreatus* to induce apoptosis, although the rates were not too high (still less than 50%). Some researchers suggest that cancer cells are cells that grow continuously with no apoptosis. However, according Jedinak and Sliva (2008) the mechanism of apoptosis

can be induced by exposure to cytotoxic agents. Kurnia YH^U (2012) reported that Cervical cancer screening was currently done by observing the proliferation and apoptosis of cells, where the proliferation of cells can be studied by destroying tissue through flow cytometry method. Apoptosis cells has the form chromatin condensation, cell shrinkage, the fragmentation of the nucleus and surrounded by a clear liquid.

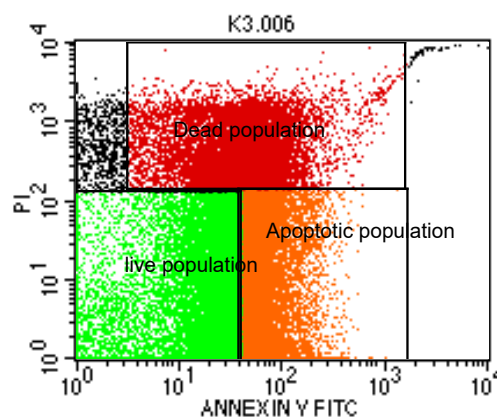


Figure 4. Results of HeLa cells analysis using flow cytometry, treatment of ethyl acetate extract of the fruit body of 100 $\mu\text{g} / \text{ml}$, (a) The cell death due to necrosis was 24.37%, (b) 34.97% of apoptotic cell death, (c) 40.15% of living cells. Spot color shows the distribution of HeLa cells.

Some bioactive compounds from plants such as buah pinang have been successfully used to induce apoptosis of MCF-7 (Meiyanto et al., 2008). Wong et al. (2007) reported that the metabolites of bioactive soluble water from the fruiting bodies of *Pleurotus tuberregium* can inhibit leukemia cancer cell (HL-60) with IC_{50} 25 $\mu\text{g} / \text{ml}$, inducing apoptosis, causing inhibition phase of cell G2-M (*G2-M arrest*), and decreasing the expression of Cdk1.

Akyuz et al. (2010) stated that some types of *Pleurotus* spp. produce flavonoid and phenolic compounds which are active as antimicrobe and anticancer. In addition, Patel and Goyal (2012), Tel et al. (2012) also stated that the various types of macroscopic fungi capable of producing bioactive compounds such as polysaccharides, proteins, fats, glycosides, alkaloids, tocoferol, phenolics, flavonoids, carotenoids, and organic acids. Helmi et al. (2016) stated that *kesembung* leaf extract contained phenol, tannin, and triterpenoids. Previous researchers reported that those compound having antimalarial activity and this extract could inhibit the plasmodium. It was assumed that phenol, tannin and triterpenoid in this

extract could inhibit the parasit. Wang et al. (2012) reported that *Linteus phellinus* polysaccharide can inhibit cell proliferation and colony formation of HepG2 cancer cells through the inhibition of cell cycle in the S phase (DNA synthesis) and induces apoptosis by increasing the release of citokrom c. Identification of bioactive metabolites have also been conducted by Tang et al. (2007), stating that the *L. edodes* and *Agaricus blazei* produce ergosterol, polyphenols, and terpenoids.

Benefits for science is to obtain information suppression of HeLa cell proliferation, which can provide new hope for cancer treatment community. The benefits of the research results was the product of *P. ostreatus* extract has the potential to inhibit the growth of HeLa cells. This product can be applied in the community with ease and in a more controlled industrial scale.

CONCLUSION

Based on the results of this study, it can be concluded that bioactive compounds from the fruit body of *P. ostreatus* showed good activity in inhibiting the proliferation of cervix cancer cells (HeLa). The highest IC₅₀ value was obtained from the treatment of the 107.59 ug/ml ethyl acetate extract of the fruit body PoMd. Kinetics of proliferation of HeLa cells showed that an increase in cell death of HeLa was in line with the length of incubation time. The mechanism of cell death by apoptosis HeLa was greater than that by necrosis.

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REFERENCES

Akyuz, M., Onganer, A. N., ERECEVIT, P., & Kirbag, S. (2010). Antimicrobial activity of some edible mushrooms in the eastern and southeast Anatolia region of Turkey. *Gazi University Journal of Science*, 23(2), 125-130.

Brady, H. J. M. (2007). *Apoptosis Methods and Protocols*. New Jersey: Humana Press Inc., Totowa.

Chakravarty, B. (2011). Trends in Mushroom cultivation and breeding. *Australian Journal of agricultural engineering*, 2(4), 102-109.

Dewick, P. M. (2002). *Medicinal Natural Products A Biosynthetic Approach*, 2nd^{ed.}. New Jersey: John Wiley & Sons, LTD.

Ekowati, N., & Mumpuni, A., & Kasiamdari, R. S. (2009). The ability of some Isolates *Lentinula edodes* in bioactive metabolites Produce Antimicrobials. *Proceedings of the National Seminar on the Role Biosistimatika in Biological Resource Management Indonesia. Purwokerto, December 12, 2009*.

Ekowati, N., Kasiamdari, R. S., Pusposendjojo, N. & Muljowati, J. S. (2012). Genetic Strain Relations *Pleurotus ostreatus* (Jacq.) P. Kumm. Java Based on Molecular Markers *Random Amplified Polymorphic DNA*. *Proceedings of the National Seminar on the Establishment of the Association of Mycology and Mycology Indonesia*, the theme "Biodiversity and Biotechnology Fungi Biological Resources" at the Faculty of Biology, University of General Sudirman, Purwokerto.

Fan, L., Pan, H., Soccol, A. T., Pandey, A., & Soccol, C. R. (2006). Advances in mushroom research in the last decade. *Food Technology and Biotechnology*, 44(3), 303-311.

Faridur, R. M., Rezaul, K. M., Farhadul, I. M., Rowshanul, H. M., & Tofazzal, H. M. (2010). Phytochemical and cytotoxic investigation on oyster mushroom (*Pleurotus ostreatus*). *International Research Journal of Pharmacy*, 1(1), 342-345.

Gregori, A, Mirjan & Pohleven, J. (2007). Cultivation Techniques and Medicinal Properties of *Pleurotus* spp. *Food Technol. Biotechnol.*, 45(3), 236-247.

Helmi, H., Afriyansyah, B. & Ekasari, W. (2016). The Effectiveness of Local Plants from Lom and Sawang Ethnics as Antimalarial Medicine. *Biosaintifika: Journal of Biology & Biology Education*, 8(2), 193-200.

Hozova, B., Kuniak, L. & Kelemenova, B. (2004). Application of β D-Glucans Isolated from Mushrooms *Pleurotus ostreatus* (Pleuran) and *Lentinula edodes* (Lentinan) for Increasing the Bioactivity of yoghurts. *Crech J. Food Sci.*, 22(6), 204-214.

Israilides, C., Kletsas, D., Arapoglou, D., Philippousis, A., Pratsinis, H., Ebringerová, A., ... & Harding, S. E. (2008). In vitro cytostatic and immunomodulatory properties of the medicinal mushroom *Lentinula edodes*. *Phytomedicine*, 15(6), 512-519.

Jedinak, A., & Sliva, D. (2008). *Pleurotus ostreatus* inhibits proliferation of human breast and colon cancer cells through p53-dependent as well as p53-independent pathway. *International Journal of Oncology*, 33(6), 1307-1313.

Kai, L. J. (2007). Secondary metabolites from higher fungi in China and their biological activity. *Drug Discov. Ther*, 1(2). 94-103.

Kurnia, I., Bintari, S. H., & Khaisuntaha, M. (2012). Tingkat keganasan kanker serviks pasien praradiasi melalui pemeriksaan AgNORs, MIB-1

- dan Cas-3. *Biosaintifika: Journal of Biology & Biology Education*, 4(2), 53-61.
- Langdon, S. P. (2003). *Methods in Molecular Medicine: Cancer cell culture, methods and protocols*. Totowa: Humana Press Inc.
- Lindequist, U., Niedermeyer, T. H., & Jülich, W. D. (2005). The pharmacological potential of mushrooms. *Evidence-Based Complementary and Alternative Medicine*, 2(3), 285-299.
- Manjunathan, J., & Kaviyarasan, V. (2010). Solvent based effectiveness of antibacterial activity of edible mushroom *Lentinus tuberregium* (Fr.). *Int. J. Pharm Tech Res*, 2, 3.
- Meiyanto, E., Susidarti, R. A., Handayani, S. & Rahmi, F. (2008). Fruit Seed Extract ethanolic Pinang (*Areca catechu* L.) were able to inhibit proliferation and trigger apoptosis of MCF-7. *Magazines Pharmaceutical Indonesia*, 19(1), 12-19.
- Patel, S., & Goyal, A. (2012). Recent developments in mushrooms as anti-cancer therapeutics: a review. *3 Biotech*, 2(1), 1-15.
- Poucheret, P., Fons, F., & Rapior, S. (2006). Biological and Pharmacological Activity of Higher Fungi: 20-Year Retrospective Analysis. *Cryptogamie, Mycologie*, 27(4), 311-333.
- Stamets, P. (2000). *Growing Gourmet and Medicinal Mushrooms*. Third Ed. Toronto: Ten Speed Press, Berkeley.
- Smith, J. E., Rowan, N. J. & Sullivan, R. (2002). *Medicinal Mushrooms: Their therapeutic properties and current medical usage with special emphasis on cancer treatments*. Cancer Research UK: University of Strathclyde.
- Tang, Y., Zhu, L., Li, H., & Li, D. (2007). Submerged culture of mushrooms in bioreactors-challenges, current state-of-the-art, and future prospects. *Food Technology and Biotechnology*, 45(3), 221.
- Tel, G., Apaydin, M., Duru, M. E., & Öztürk, M. (2012). Antioxidant and cholinesterase inhibition activities of three *Tricholoma* species with total phenolic and flavonoid contents: the edible mushrooms from Anatolia. *Food Analytical Methods*, 5(3), 495-504.
- Tjindarbumi, D., & Mangunkusumo, R. (2002). Cancer in Indonesia, present and future. *Japanese Journal of Clinical Oncology*, 32(suppl 1), S17-S21.
- Wang, G., Dong, L., Zhang, Y., Ji, Y., Xiang, W., & Zhao, M. (2012). Polysaccharides from *Phellinus linteus* inhibit cell growth and invasion and induce apoptosis in HepG2 human hepatocellular carcinoma cells. *Biologia*, 67(1), 247-254.
- Wasser, S. P. (2002). Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Applied microbiology and biotechnology*, 60(3), 258-274.
- Wijayanti, N. K. (2007). Antiproliferative activity test ethanolic leaf extract gombong hibiscus (*Hibiscus similis* L.) against cervical cancer cells (HeLa). *Report of Research, Faculty of Pharmacy, University of Gadjah Mada, Yogyakarta*.
- Wong, S. M., Wong, K. K., Chiu, L. C. M., & Cheung, P. C. K. (2007). Non-starch polysaccharides from different developmental stages of *Pleurotus tuber-regium* inhibited the growth of human acute promyelocytic leukemia HL-60 cells by cell-cycle arrest and/or apoptotic induction. *Carbohydrate Polymers*, 68(2), 206-217.