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

✉Nuraeni Ekowati, Aris Mumpuni, Juni Safitri Muljowati

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Faculty of Biology, University of Jenderal Soedirman, Purwokerto, Indonesia

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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-dimetiltiazol-2-yl) -2.5-diphenyl tertrazolium 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 µg / ml. HeLa cell proliferation kinetics analysis showed that the application of bioactive compounds 100 µg / 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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✉ Correspondence Author:
Jl Dr. Soeparno No. 63, Grendeng, Purwokerto, Jawa Tengah 53122
E-mail: nuraeniekowati@yahoo.com

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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 (Tjindarbumi & Cipto, 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 Kaviyarasan (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 (ß-glucan) and terpenoids. Pleuran (ß-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-

amining anticancer ability may lead to discover superior strains. It is necessary to asses the potency of each strain for the production of bioactive compounds and examine the compounds in inhibiting the cervical cancer cell proliferation activity. The research objectives are: 1) to obtain the extract of bioactive compounds from the medium filtrate and fruit body *of P. ostreatus* potent in inhibiting cervical cancer cell proliferation (HeLa cells), 2) to evaluate the kinetics of proliferation of HeLa cells, 3) to determine the mechanism of HeLa cell death by administering bioactive compounds. 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.

METHODS

The method was experimental, to test the use of cytotoxic treatment dose (in µg/ml) 0; 31.25; 62.50; 125; 250; 500; 1,000. Furthermore, the living cell and the value of *Inhibition Concentration 50* (IC_{50}) were counted.

Tests were carried out on the three strains selected based on the analysis of genetic variation. Bioactive compounds used were derived from culture filtrate and fruitbody extract. The parameters measured were the anticancer activity (cytotoxic) in HeLa cells (the optimum concentration that can suppress the growth of HeLa cells), HeLa cell proliferation kinetics and mechanisms of HeLa cell apoptosis.

The research procedure begins with making the culture medium for fermentation *P. ostreatus,* cultivated for 28 days using Kauffman medium (KM) with the following composition: 10.0 g malt extract; yeast extract 5.0 g; maltose 5.0 g; peptone 1.5 g; KH_2PO_4 0.25 g; $MgSO_4$ 0.5 g; $Ca(NO_3)_2$ 0.5 g; KCl 0.5 g; $Fe(NO_3)_3 9H_2O$ 723.5 mg; $ZnSO_4 7H_2O$ 439.8 mg; $MnSO_4 4H_2O$ 203.0 mg; up to 1.0 liters of distilled water. The medium was inoculated with *P. ostreatus* and incubated in room temperature at 150 rpm orbital shaker for 28 days. After 28 days the biomass of mycelium harvested and separated from the culture filtrate and the bioactive metabolites of each treatment were extracted. The culture was then filtered using Whatman filter paper no. 41, in a Buchner funnel and flask with vacuum pumps. Medium filtrate obtained was extracted twice in succession with chloroform (first extraction) and ethyl acetate (second extraction). At the first extraction, 50 ml of the medium filtrate extracted with 50 ml chloroform in a separating funnel (repeated 2X), shaken with hands for 5 minutes, put on the stand for ± 5 minutes until chloroform and the filtrate were separated. The filtrate was then added with 50 ml of ethyl acetate (repeated 2X), shaken with hands for 10 minutes, put on the stand for ± 5 minutes until ethyl acetate and the filtrate were separated. The extract of both chloroform and ethyl acetate obtained were evaporated using a rotary evaporator at 45 ^{0} C. After evaporation, the extracts were powdered with an analytical balance. The dried extracts were then stored at 5^0 C until use. When in use the extracts were weighted according to the dose and diluted with 5% dimethylsulfoxide (DMSO).

The production and extraction of secondary metabolites from the fruiting bodies *of P. ostreatus* was done by drying the fruit body using a freeze dryer (2 x 24 hours) then pulverized to a powder. Fruit body powder was extracted using 50 ml of chloroform and shaken using an orbital shaker for 24 hours. Chloroform extract and powder are separated by centrifugation at 4500 rpm for 15 minutes, then powder which had separated was extracted again with 50 ml of chloroform (extraction was done twice in a row). Chloroform extract of the first and second extraction are mixed, the solvent is evaporated with a rotary evaporator. The second extraction using ethyl acetate was carried out in the same manner with that of chloroform. The obtained dry extract were then powdered to find the dry weight of the extract. The extracts were then stored at 5^0 C until use.

Furthermore, the cytotoxic test using ethyl acetate and chloroform extracts on HeLa cancer cells were performed using methods of Langdon (2003) proceed by diluting bioactive compounds extract of each treatment into concentrations (in µg / ml) : 0; 31.25; 62.50; 125; 250; 500 and 1,000. Cytotoxicity assay was performed with MTT method [3- (4,5-dimetiltiazol-2-yl) -2,5-diphenyl tertrazolium bromide]. A total of 100 µL of the compound extract from *P. ostreatus* was loaded into 96-well plate. A part of the wells which already contain the extract was then added with 100 µL of HeLa cells and another part with 100 µL of RPMI medium. Other wells were filled to 200 µL of the medium as control treatment, the other part filled with 100 µL medium along with 100 µL HeLa cells. Furthermore, the cells were incubated in an incubator with 5% carbon dioxide flow at 37 ^{0} C for 24 hours. At the end of incubation, to each of the well was added 10 µL of MTT 2.5 µg/ml in PBS. After 4 hours MTT reaction was stopped by addition of 100 µL reagent-stopper Acid Isopropanol, then incubated 12 hours at room temperature. Absorbance were

red by *ELISA reader* at a wavelength of 550 nm. Results of absorbance reading were converted into percentages life of HeLa cells to obtained IC_{50} 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 CO_2 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:

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

Life percentage of cell data were converted to the IC_{50} (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% (IC_{50} . The results of cytotoxic bioactive compound assay of *P. ostreatus* shown in Table 1.

Analysis to determine the value of IC_{50} on HeLa cells showed that bioactive metabolites from *P. ostreatus* inhibited the growth of HeLa cells with IC_{50} values varied (Table 1). The lowest IC_{50} value of the ethyl acetate extract was 107.59 ug/ml. The smaller the IC_{50} values indicated the more active extracts to kill HeLa cells. *P.ostreatus* isolated from Madiun resulted in the lowest IC_{50}. 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 IC_{50} of the fruit body

Table 1. IC_{50} 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 Ml	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 Ml	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 Ml *(P. ostreatus* from Malang), Po P *(P. ostreatus* from Purwokerto)

water extract of *L. edodes* was 119 ug / ml, and for the water extract of mycelium was > 1000 ug / ml.

The regression analysis of the best two extract treatment of the fruit body of *P. ostreatus* shown in Figures 1 and 2.

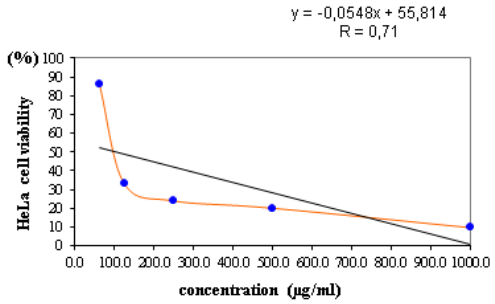


Figure 1. Relationship between the concentration of the ethyl acetate extract of fruitbody of Po Md and HeLa cell viability (IC $_{50}$ = 107.59 µg/ml).

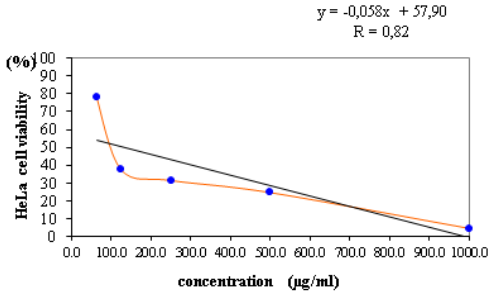


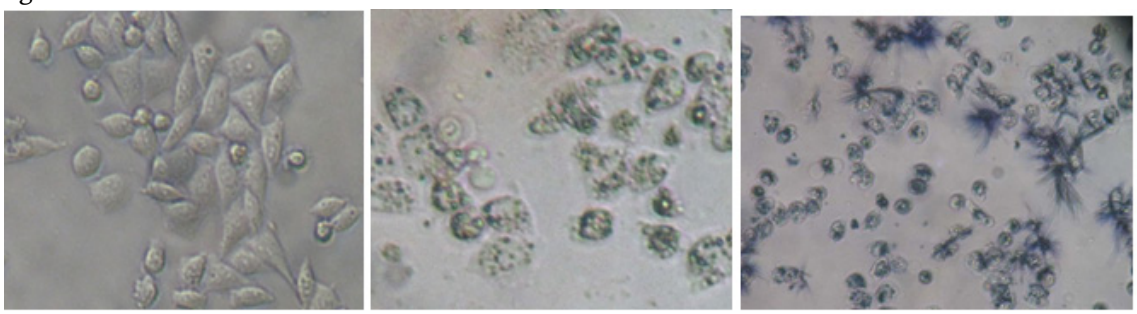
Figure 2. Relationship between the concentration of ethyl acetate extract of the fruitbody of Po Ml and HeLa cell viability (IC $_{50}$ = 136.21 µg/ml).

Cytotoxic test results showed that the higher level of bioactive metabolites extract the lower percentage of living HeLa cells. Meiyanto et al. (2008) showed that ethanol extract of buah pinang *(Areca catechu)* inhibits breast cancer cells (MCF-7) with IC_{50} 77 ug/ml compare to that of arekolin compound 180 ug/ml. Israilides et al. (2008) used the extract of the fruit body of *L. edodes* which were tested on MCF-7 cells resulting IC_{50} value of 73 ug/ml. Wijayanti (2007) assayed ethanol extracts of gombong hibiscus leaves *(Hibiscus similis* L.) on HeLa cells resulting IC_{50} value of 138.4 µg/ml. According to Faridur et al (2010); Patel and Goyal (2012) reported a group of bioactive metabolites terpenoids, steroids, phenolics, flavonoids and alkaloids as potential anticancer compounds.

Observation on extract of *P. ostreatus* on the viability of HeLa cells were done using the *ELISA Reader* to count the number of living cells, as well as observation of HeLa cell morphology using *inverted microscope* (Figure 3).

Figure 3(a) showed a healthy HeLa cells, the cells appear normal and were attached to the base of complete RPMI medium. Figure 3 (b) showed the morphology of HeLa cells that were not normal due to exposure to bioactive compounds extract of *P. ostreatus,* the cells turned into spherical and splitted into smaller spherical forms, as well as cell shrinkage occurs. HeLa cells that grow normally will attach firmly to the base of the medium and shaped like a leaf after incubation time of 24 hours. If these cells are round and not attached to the medium base, it indicates that the cell was not healthy. Figure 3 (c) showed HeLa cells after treatment MTT, the formazan formation appears to occur in healthy cells, because of their enzymatic reactions in healthy cells, whereas the dead HeLa cells did not perform enzymatic reac- tion and formazan were not constructed. The picture showed that there were more dead cells than that of the healthy ones.

Observation of HeLa cell proliferation kinetics by application of bioactive compounds was conducted during the incubation period 0 h, 24 h, 48 h and 72 h. The results showed that the percentage of living HeLa cells decreased with the increase of incubation time. Nearly all HeLa cells



(a) (b) (c)

Figure 3. Morphology of HeLa cell cytotoxic assay results. (a) healthy HeLa cells (normal), (b) abnormal HeLa cells because the chloroform extract Po Md fruiting bodies with a concentration of 125 µg / ml (c), HeLa cells after treatment of MTT.

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 life 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 fluorescense 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 et al. (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 throught flow cytometrymethod. Apoptosis cells has the form chromatin condensation, cell shrinkage, the fragmentation of the nucleus and surrounded by a clear liquid.

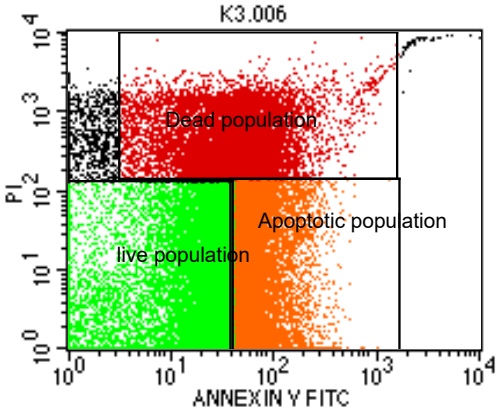


Figure 4. Results of HeLa cells analysis using flow cytometry, treatment of ethyl acetate extract of the fruit body of 100 µg / ml, (a) The cell death due to necrosis was 24.37%, (b) 34.97% of apoptotic cell death, (c) 40.15% of living cells. Spot color shows the distribution of HeLa cells.

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 µg/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 el 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 IC_{50} 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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