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The Cytotoxic and Apoptosis Effects of Chloroform Extracts of *Auricularia auricula* on Cervical Cancer Cells

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Abstract

Auricularia auricula is an edible mushroom cultivated in Indonesia that has been known to have potential properties of bioactive compounds than can be used for medicinal purpose. This study aimed to examine the cytotoxic and apoptosis effect of chloroform extract of *A. auricula* on cervical cancer cells in vitro. The research design was in vitro experimental research. Cytotoxic tests was using an MTT [3-(4,5-dimetiltiazol-2-yl)-2.5-diphenyl tertrazolium bromide] assay and apoptosis test was using double staining method. Test of bioactive compounds was carried out using GCMS. Cytotoxic effect were analyzed by linear regression and apoptosis test was analyzed descriptively. Chloroform extract of mycelium *A. auricula* showed the best results with IC_{50} = 264.87 µg/ml. An important finding obtained after the double staining process was that chloroform extract of *A. auricula* can induce HeLa cells death by apoptosis. GCMS test results showed that the extracts containing limonene and piperidinone which are the anticancer bioactive compounds. In conclusion, the chloroform extracts of *A. auricula* has the potential to inhibit the growth of cervical cancer cells. The benefit of this study are expected to provide information about the anticancer potential of extract of *A. auricula* against cervical cancer cells, thus contributing to the development of alternative anticancer treatments from natural product.

How to Cite

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INTRODUCTION

The use of mushrooms as an alternative food source has grown rapidly in the past decade. Some mushrooms have been widely consumed because they contain bioactive compounds and high nutritional value (Lindequist, 2013). *Auricularia auricula* is one of the mushrooms that have long been cultivated in Indonesia. The fruiting body of *A. auricula* contains primary metabolites such as polysaccharides, and secondary metabolites such as alkaloids, flavonoids, triterpenoids, saponins (Essien et al., 2015). The use of mushrooms as a drug or supplement in the treatment of diseases due to their bioactive compounds continues to increase (Rathee et al., 2012). One of the roles of bioactive compounds contained in *A. auricula* which has been widely studied is as an anticancer.

Cancer is a disease caused by loss of cell control function to cycle regulation and cell homeostatic function, cells will proliferate continuously resulting in abnormal tissue growth (Cancer Chemoprevention Research Center, 2014). Cervical cancer is the most common type of cancer in women in Indonesia. In 2013, cervical cancer had the highest prevalence in Indonesia at 0.8% followed by breast cancer with a prevalence of 0.5% (Depkes, 2015).

Generally, cancer cells develop from normal cells due to DNA damage. Damaged DNA causes the apoptosis mechanism to be disrupted (Sudhakar, 2009). The main function of apoptosis cell is to maintain the tissue homeostasis, a balance between cell proliferation and cell death (Bai & Zhu, 2006). Apoptosis is described as a process of active cell death due to the results of independent cellular disassembly without any inflammation. While necrosis is described as a passive cell death due to environmental disorders by the release of cellular contents which causes uncontrolled inflammation (Susan, 2005). In cancer, some abnormal cells succeed in avoiding the apoptosis process. Rebecca (2011) divided apoptosis avoidance mechanisms as follow: 1) disruption of the balance of pro-apoptosis and anti-apoptosis proteins, 2) reduction of caspases function and 3) disruption the death receptor signals. The strategy of cancer treatment is to restore the apoptosis signaling pathway to a normal state so that it has the potential to reduce and eliminate cancer cells.

Unfortunately, the use of anticancer drugs such as chemical drugs or chemotherapy does not only kill the cancer cells, but also damage the other cells, which can cause a decrease in immune function or even death (Li et al., 2008). The using of natural product is relatively safer because the side effects are relatively small when compared to chemical drugs, chemotherapy, surgery and radiation. Anticancer compounds play an important roles including inducing Reactive Oxygen Species (ROS), mitotic kinase inhibitors, anti-mitosis, angiogenesis inhibitors, topoisomerase inhibitors, cause the apoptosis, and inhibit the cancer proliferation (Patel, 2012). The proving of whether a bioactive compound can be toxic or inhibiting the growth of cancer cells can be done using cytotoxic test and antiproliferative test in vitro. HeLa cells are cervical cancer cells caused by *Human papillomavirus* infection (HPV 18) (Landry et al., 2013).

Several studies have shown that the ethanol extract of the fruiting body of *A. auricula* has cytotoxic and antiproliferative effects on lymphoid tumor cells (P388D1) and sarcoma 180 (Ahsanur et al., 2011). Immunohistochemical results show that *A. auricula* extract can induce apoptosis in S-180 tumor cells by regulating Bax and down-regulating Bcl-2 (Xiaoqing et al., 2015).

This study aimed to determine the cytotoxic and apoptosis effects of chloroform extract of *A. auricula* on cervical cancer cells (HeLa) in vitro. Cytotoxic tests can be carried out using the MTT (3-(4,5-dimetiltiazol-2-yl)-2.5-diphenyl tertrazolium bromide) assay method. Apoptosis cell was observed after staining using double staining method. The results of this study are expected to provide information about the anticancer potential of chloroform extract of *A. auricula* against cervical cancer cells (HeLa), thus contributing to the development of alternative anticancer treatments from natural product.

METHODS

The research method used was in vitro experiment. The treatment tested the effect of chloroform extract of fruiting body and mycelium of *A. auricula* against HeLa cell growth. The parameters measured were cytotoxic activity tested by MTT assay method and apoptosis induction which was observed after the staining using double staining method.

The procedure of the study began with the preparation of the sample (the fruiting body and mycelium *A. auricula*). The fruiting body of the mushroom was obtained from mushroom cultivation in Purwokerto. Mycelium was obtained by making mycelium culture of *A. auricula*, using a MCM (Mushroom Complex Medium) medium with composition of 20 g glucose; 2 g peptone; 2 g Yeast Extract; 0.46 g KH_2PO_4; 0.5

g $MgSO_4.7H_2O$; 1 g K_2HPO_4. The prepared mushroom mycelium was inoculated into MCM medium and incubated at room temperature in orbital shaker for 28 days. After 28 days, the mycelium biomass was harvested and filtered with whatman paper no. 41 using a filter funnel and vacuum. Mycelia and fruiting bodies were oven-dried at 50°C, then mashed into powder using a blender.

Fruiting body and mycelium powder were extracted using chloroform solvent by maceration. Maceration was done by soaking the sample with 50ml of chloroform and shaked using orbital shaker for 24 hours. The supernatant and powder were separated using a 4500 rpm centrifuge for 15 minutes, the separated powder was macerated again with 50 ml of chloroform as the second extract. The first and second supernatants were combined and evaporated using a hot plate until a thick chloroform extract was obtained.

Cytotoxic assay of chloroform extract of *A. auricula* fruiting body and mycelium against HeLa cells was carried out using MTT assay method, testing each extract using a concentration of 62.5; 125; 250; 500 and 1000 µg/ml. The first step was planting of 100 µL of Hela cells in RPMI medium in microplate 96-wells, some wells were only filled by RPMI medium as a control. Cell culture was incubated for 24 hours in an incubator at 37°C and 5% CO_2. After incubation, culture medium in wells were removed. The wells of cell control and medium control were added 100 µL of new RPMI medium, while the wells of treatment were added 100 µL of suspension from RPMI medium and the extract according to each treatment concentration. Cell culture was re-incubated for 24 hours. After the incubation, medium were removed and replaced with a new one, then at each well 10 µL of MTT reagent (3-(4-5 dimethylthiazol2-yl) -2, 5- diphenyl tetrazolium bromide) were added and incubated for 4 hours. Then the 50 µL of reagent stopper (sodium dodecyl sulfate 10%) were added. Microplate was wrapped in aluminum foil, and incubated at room temperature overnight. In the last stage, absorbance of the cell was read using an ELISA reader at a wavelength of 595 nm. Results obtained were used to calculate the percentage of living cells and IC_{50} values for each treatment.

Apoptosis test was done after the double staining method using acridine orange/ethidium bromide (AO/EB) dye. The treatments used IC_{50} concentration based on previous cytotoxic test results. The first step was planting 1mL of Hela cells in RPMI medium to coverslip which was placed in microplate 6-well and incubated for 24 hours. After incubation, medium were removed and replaced with 1 mL of new medium for control and 1 mL of medium with suspension of chloroform extract of *A. auricula* in IC_{50} concentration. After 24 hours of incubation, medium were removed again and rinsed by Phosphate-buffered saline (PBS). Then coverlip was removed and placed on a object glass, then 10 µL 1x Work Solution (AO/EB) was added and let stand for 5 minutes. The observation of the mechanism of death cell was observed using a fluorescent microscope.

The bioactive compounds testing was carried out using the GC-MS QP2010 Shimadzu. The column used by Rtx-5MS (Fused Silica) using Helium carrier gas. The mass spectra results were compared with the National Institute of Standards and Technology (NIST) database, NIST 27, NIST and WILEY 7 database.

The absorbance data obtained from the cytotoxic test was converted into percent of living cells by below formula.

$$\text{Percent of living cell} = \frac{\text{absorbance of treatment - absorbance of media}}{\text{absorbance of control- absorbance of media}} \times 100\%$$

Data on the percentage of living cells was converted to IC_{50} using linear regression analysis. Morphology of cell death in the apoptosis test after the double staining method was analyzed descriptively.

RESULTS AND DISCUSSION

The extraction of bioactive compounds from the fruiting body and mycelium of *A. auricula* using chloroform resulted in extracts with dense and dark brown colour. The testing of cytotoxic effects can be observed directly using an inverted microscope. The morphological observations of HeLa cancer cells after the treatment by chloroform extract of *A. auricula* and MTT reagent can be seen in Figure 1.

The form of a healthy HeLa cell was long and large. Abnormal cells had a smaller size and was blebbing in cell membrane while dead cells appear fragmented and destroyed. HeLa cells were inhibited due to an induction by chloroform extract of *A. auricula* that had apoptosis mechanisms. That mechanisms caused changes in cell morphology. Apoptosis can occur in several stages including shrinkage of cell density, condensation and fragmentation ofcell chromatin and cell nucleus fragmentation (Wyllie, 2010). The difference in cell morphology between living cells and dead cells was seen after the addition of MTT. Living cells appear to have a larger size and the way they bind the MTT looks like some hairs throughout the cell surface, while in dead cells it

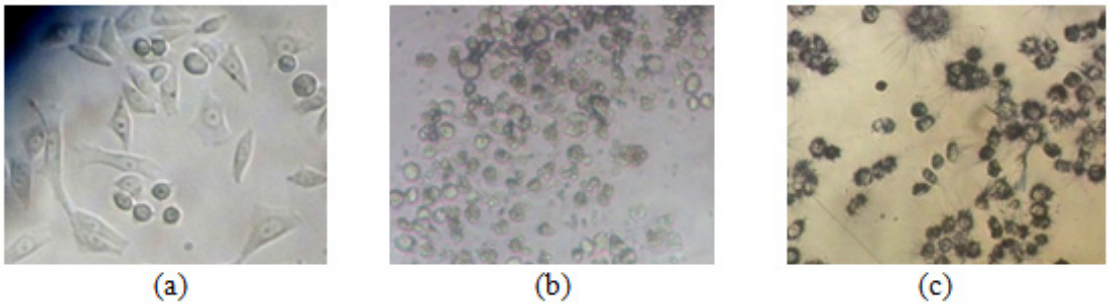
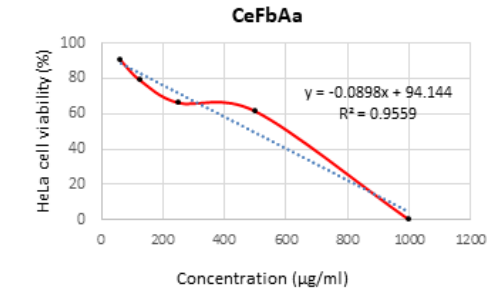


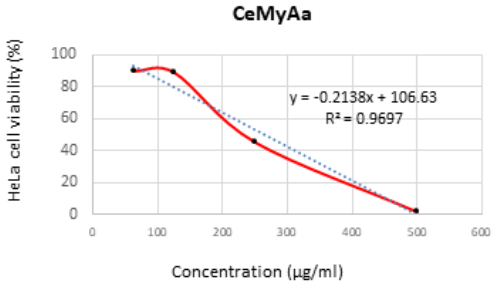
Figure 1. Morphology of HeLa cells in MTT assay test. (a) HeLa cells are healthy (normal) before MTT assay (b) Abnormal HeLa cells due to chloroform extract of mycelium of *A. auricula* at concentration 1000 µg / ml. (c) HeLa cells after MTT tested.

appears to have a smaller size and does not bind the MTT. Mitochondrial dehydrogenase activity in living cells has the ability to convert MTT to formazan crystals (Chapdelaine, 2001). Formazan crystal concentrations can be determined by spectrophotometry and directly proportional to the number of living cells.

Cytotoxic test using the MTT assay method was used to calculate the ability of a bioactive compound to inhibit cancer cell growth determined by IC_{50} value (concentration that can inhibit cell growth by 50%). IC_{50} value was obtained from calculations with linear regression analysis of the data of the relationship between the percentage of living cells and the concentration of *A. auricula* extracts. The results of linear regression analysis can be seen in Figure 2.



a)



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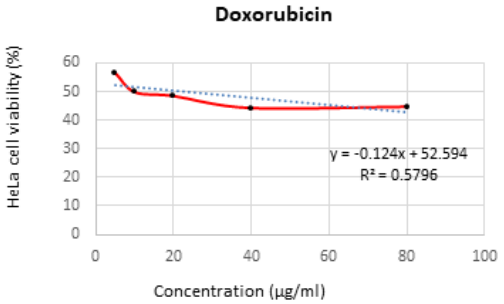

c)

Figure 2. Graphic regression analysis of chloroform extract of *A. auricula* concentration on the percentage of HeLa cell viability. a) CeFbAa, b) CeMyAa, c) Doxorubicin.

Based on the results of linear regression analysis, the regression equation used to calculate IC_{50} values is as seen in Table 1.

Table 1. Results of calculating IC_{50} values

Treatment	IC_{50} (µg/ml)
CeFbAa	491.58
CeMyAa	264.87
Doxorubicin	15.80

Description: CeFbAa (Chloroform extract of fruiting body of *A. auricula*), CeMyAa (Chloroform extract of mycelium of *A. auricula*)

The results of the analysis showed that chloroform extract of *A. auricula* could inhibit the growth of HeLa cells with the lowest IC_{50} value was CeMyAa 264.87 µg/ml. Positive control doxorubicin had IC_{50} 15.80 µg / ml.

Research using the same method has been widely reported. The cytotoxic tests done by Ekowati (2017) using HeLa cells showed IC_{50} values of chloroform extract of *P. ostreatus* fruiting body was 218.87 µg/ml. Israilides et al. (2008) used the

extracts of *L. edodes* fruiting body against MCF-7 cells and got IC_{50} by 73 µg/ml. The tested bioactive compound was found to have anticancer activity if the IC_{50} value was less than 1000 µg/mL after a 24-hour contact with cancer cells. The smaller the IC_{50} value of a bioactive compound, the more effective it will bein inhibiting cancer cell growth (Meiyanto, 2008)

The apoptosis effect of chloroform extract of *A. auricula* on HeLa cells can be observed by observing the cells morphology after double staining test. The morphology of living HeLa cells and dead HeLa cells is shown in the results of the double staining test using AO/EB is shown in Figure 3.

The results of the observation showed a color difference between living cells and dead cells due to apoptosis and necrosis. In cell control, all cells were green which shows that all cells were living. In CeFbAa and CeMyAa, the some cells were green and the others were red indicating that, there were living cells and cells that die due to the apoptotis; where as in doxorubicin treatment, there were many red colored cells with a ruptured membrane that shows dead cells due to the necrosis.

Gewies (2003) argued that the process of cancer cell death through the mechanism of apoptosis is better than through necrosis because it does not cause any inflammatory response. Reed (2000) described that the characteristics of apoptosis cells are the occurrence of chromatin condensation and nucleus fragmentation, shrinkage of plasma membranes, and cell shrinkage. Cells break into small fragments surrounded by membranes (apoptosis bodies), which are then cleared by phagocytosis without causing an inflammatory response. Lee, et al. (2018) explained that cell death by necrosis begins with cell swelling resulting in cell membrane rupture and release of cellular cytoplasmic contents into the extracellular space. The molecules that are released trigger the work of immune cells, which can cause inflammatory reactions in the area around cell death.

There have been many studies of bioactive compounds from mushroom that can induce apoptosis of cancer cells. Ethyl acetate extract of *P. ostreatus* fruiting body can induce apoptosis in HeLa cells (Ekowati, 2017). Immunohistochemical results showed that *A. auricula* extract could induce apoptosis in S-180 tumor cells by regulating Bax and down-regulating Bcl-2 (Xiaoqing et al., 2015).

The test material compound that was identified using GC-MS was CeMyAa, considering that this extract had the best cytotoxic effect in the cytotoxic MTT test with the lowest IC_{50} value. The results of GC-MS analysis of CeMyAa samples detected 52 peaks in the chromatogram which were observed at a retention time of 5,892 to 54,948 minutes with different relative concentrations. Based on data analysis of the compound content of EkMiAa extract, there were several active compounds contained with anticancer roles, namely Piperidinone (2.47%) and Limonene (1.93%).

Piperidinone is an alkaloid group compound. Research by Bezerra et al. (2006) regarding the effect of extract of *Annona muricata* leaf on MCF-7 cancer cell growth, showed that piperidinone is one of the bioactive compound able to inhibit MCF-7 cancer cells. The results of research conducted by Liu et al. (2017) also proved that piperidinone from curcumin can induce ROS-mediated apoptosis in A549 lung cancer cells. Limonene is a terpenoid derivative which belongs to the monoterpene class. Jessica, et al. (2013) explained that limonene can reduce the expression of D1 cyclin in breast tumors which can cause cell cycles to stop and reduce cell proliferation. Research conducted by Jia, et al. (2013) reported that D-limonene can induce apoptosis through mitochondrial death pathway by inc-

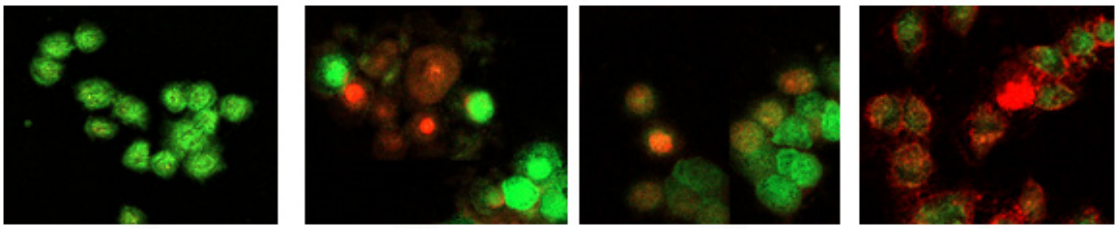

Figure 3. HeLa cancer cells after being stained with acridine orange/ethidium bromide. Cells that are green are living cells (normal), cells undergoing apoptosis are orange/red, while cells that are necrosis are red with membranes ruptured. (a) cell control, (b) CeFbAa (c) CeMyAa (d) positive control of doxorubicin.

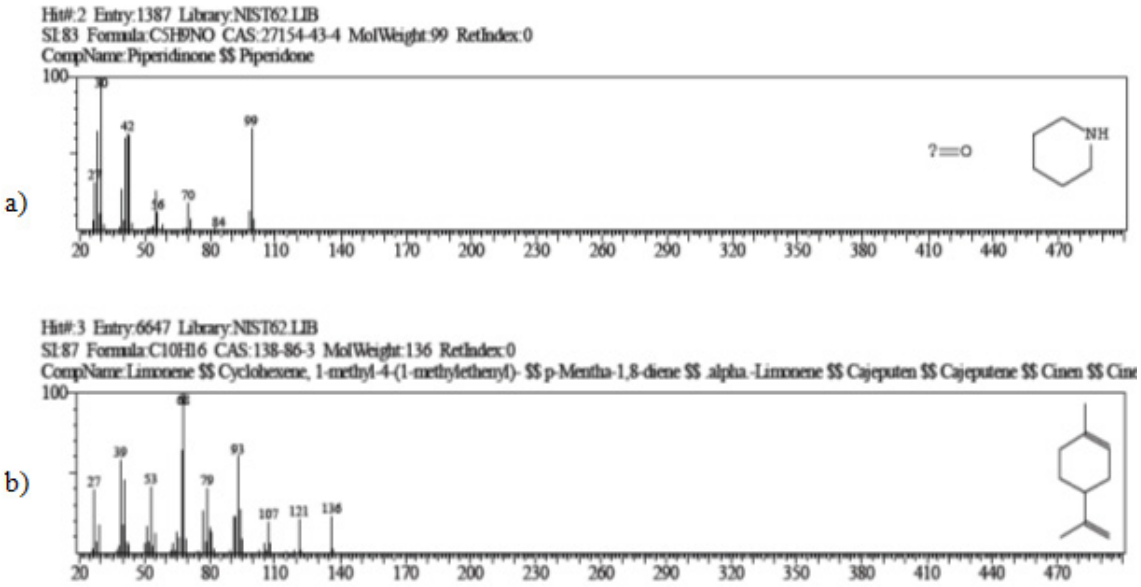

Figure 4. Chromatogram of CeMyAa by GCMS result. a) peak of Piperidinone, b) peak of Limonene

reasing Bax protein expression, cytochrome-c release from mitochondria, and caspase-3 and 9 cleavage.

The results of this study can be used to provide information about the anticancer potential of chloroform extract of *A. auricula* against cervical cancer cells (HeLa), thus contributing to the development of alternative anticancer treatments from natural product.

CONCLUSION

Based on the results of the study, it can be concluded that bioactive compounds from the chloroform extracts of the fruiting body and mycelium of *A. auricula* can inhibit the growth of cervical cancer cells (HeLa). The best IC_{50} values were obtained from chloroform extracts of *A. auricula* mycelium with a concentration of 264.87 µg/ml. Observation of HeLa cell morphology based on double staining test showed that chloroform extracts of *A. auricula* can induce cell death through apoptosis mechanism. chloroform extracts of *A. auricula* contain anticancer bioactive compounds includes piperidinone and limonene as shown by GCMS test.

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