Effects of Ethyl Acetate Extract of Jew’s Ear Mushrooms (Auricularia auricula) on Cytotoxic and Apoptosis of Cervical Cancer Cells (HeLa)

by Aris Mumpuni

Effects of Ethyl Acetate Extract of Jew's Ear Mushrooms (*Auricularia auricula*) on Cytotoxic and Apoptosis of Cervical Cancer Cells (HeLa)

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Abstract. The increasing number of people living with cervical cancer encourages the search for bioactive compounds from natural ingredients such as macroscopic fungi that have the potential to induce apoptosis. Jew's ear fungus (*Auricularia auricula*) is an edible mushroom that is also commonly used by society for medicinal purposes including curing cancer. The study aimed to examine the cytotoxic, antiproliferative, and apoptotic effects of ethyl acetate extract from the mushroom on cervical cancer cells (HeLa) *in vitro*. The research consisted of experiments. Cytotoxic and antiproliferative tests were carried out using MTT (3- (4-5 dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide) assay, while apoptosis test was the double staining method using acridine orange/ethidium bromide. The data of cytotoxic and antiproliferative effects were analyzed with linear regression, whereas apoptosis test result was analyzed descriptively. The results showed that the best cytotoxic effect was ethyl acetate extract of mushroom fruit body with IC_{50} of 538 µg/ml. Antiproliferative analysis of HeLa cells showed the best inhibitory effect of the mushroom fruit body extract at 24-hour incubation time. The apoptosis test showed that HeLa cell death through apoptosis mechanisms was higher than by necrosis.

1. Introduction

Auricularia auricula (Bull.) J. Schröt. is an edible mushroom found throughout the world. It grows on wood with ear-like shape, and brown fruiting body. The fungus is useful as a medicine due to its bioactive compounds. It has been used as a traditional medicine in China, but further scientific evidence to support it is still required. The dichloromethane extract of *A. auricula* has the potential to be anti-inflammatory and inhibits the production of Nitric Oxide (NO), Interleukin 6 (IL-6), Tumor Necrosis Factor- α (TNF-α), and Interleukin-1β (IL-1β) in macrophages [1].

A. auricula has been extensively studied for its use as a medicine and a source of new chemicals with potential therapeutic effects. In a study of water extract from seven edible fungal species tested for antitumor activity against Sarcoma 180 transplantation in Swiss albino mice, the results showed that *A. auricula* significantly inhibited the growth of transplanted tumors [2]. Furthermore, studies of the hypoglycemic effect of water-soluble polysaccharides from *A. auricula* fruit bodies were investigated in genetic diabetic rats (KK-Ay) ages 10-14 weeks. The rats showed a decrease in plasma glucose, insulin, and urine glucose [3]. Other studies about the effects of *A. auricula* on anticoagulant aggregation have been investigated [4]). No results, however, concerning the anticancer effects of *A. auricula* ethyl acetate extract from Indonesia have been reported.

The potential of bioactive compounds of *A. auricula* as an anticancer can be detected through testing the cancer cells directly [5, 6]. One type of cancer cell used for *in vitro* testing is cervical cancer cells (HeLa cells). Cervical cancer is the first incidence of cancer in women, and no treatment method is yet available [7].

The most widely used cancer treatment is chemotherapy. This method, however, has side effects, one of which is the high damage of healthy cells. Thus, through this study, it is expected to reveal the bioactive metabolites potential of *A. auricula* in suppressing HeLa cell growth. Bioactive metabolites of *A. auricula* can be isolated from the fruiting bodies, which are obtained by cultivation of the fungus on solid medium. The metabolites can also be isolated from mycelium biomass and culture filtrate by liquid medium cultivation [8]. The β-glucan is the fungus bioactive metabolite which has been studied for its therapeutic potential [5], however, other bioactive metabolites require further studies. Thus, it is necessary to isolate and identify the classes of bioactive metabolites produced by *A. auricula* and test their potential. The study aimed to examine the cytotoxic, antiproliferative, and apoptotic effects of ethyl acetate extract from ear mushroom on cervical cancer cells (HeLa) in vitro.

2. Methods

The method used was *in vitro* experimental research. Four treatments were use in the experiments, i.e., fruit body, mycelium, and culture filtrate of *A. auricula*, and Doxorubicin. Anticancer activity test was applied to cervical cancer cells (HeLa). Cytotoxic and antiproliferative tests were carried out using 3- (4-5 dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay. A double staining method using acridine orange and ethidium bromide dyes was applied for apoptosis test. Cytotoxic and antiproliferative data were analyzed by linear regression whereas descriptive analysis was for apoptosis test. Cytotoxic tests were used to treat extracts of secondary metabolites with concentrations of 62.50, 125, 250, 500 and 1,000 µg/ml. The variables observed were living cells, and Inhibition Concentration 50 (IC_{50}) values, cervical cancer cell (HeLa) morphology, cancer cell proliferation kinetics, and cancer cell death mechanisms.

The experiments included (1) making the Roswell Park Memorial Institute (RPMI) medium for cancer cells, (2) preparing and multiplicating cervical cancer cells (HeLa), (3) diluting the bioactive compound extract for treatments with the given concentration series, and (4) testing the cytotoxic activity of each cancer cell.

Bioactive metabolite testing procedure [9]

A total of 100 µl of the extract was taken from the treated medium prepared with the varied metabolite concentrations, and transferred to a 96-well plate containing 100 µL of HeLa cells aged 24 hours. Each 96-well plate was used to test four treatments with each using five series of extract concentrations (three replicates). The wells without extract were used for cell and medium controls (three replicates each). For medium control, 200 µl of complete medium was filled into well, while for cell control, 100 µl of complete medium was filled with 100 µl of HeLa cells (10^4 cells). Furthermore, 96-well plates that already contained cells and extracts were incubated in an incubator with a flow of 5% carbon dioxide at 37° C for 24 hours.

The cells in each extract concentration were observed and photographed under an inverted microscope after incubation. By reversing the plate, the media in the 96-well plate were removed. The remnants in the well were washed with PBS 1x. In each well, 10 µl (with a concentration of 0.5 mg/ml in PBS) MTT reagents were added, and 100 µl complete medium for each well including the control medium, were incubated for 4 hours in a 5% CO_2 incubator. After 4 hours, it was observed under an inverted microscope. When formazan was formed, the observation was stopped by adding 100 µl of SDS 10% stopper reagent in 0.1 N HCl. The enzymes of the living cells reacted with MTT demonstrating purple or blue color in the cell. The plate was wrapped in paper and incubated overnight at room temperature. The following procedure was observing the plate with ELISA reader. The plate was opened and placed on the ELISA reader with a wavelength of 595 nm (λ = 550-600

nm). The readable absorbance was converted into the percentage of HeLa living cell. Data were analyzed by regression analysis to obtain Inhibition Concentration 50 (IC_{50}) values.

Data analysis

The living cells were counted for cytotoxicity analysis. The absorption responses of the cytotoxicity test were converted into percent of cancer cell life with the following formula:

$$Living\ cell = \frac{\text{absorbance of treatment} - \text{absorbance of control medium}}{\text{absorbance of control cell} - \text{absorbance of control medium}}\ x\ 100\%$$

Based on the results of living cells, the IC_{50} values were calculated using the Statistics for Window program.

3. Results

IC_{50} values of anticancer tests showed various results. The best value was obtained from ethyl acetate extract of the fruit body (IC_{50} value of 538 µg/mL), followed by ethyl acetate extract of mycelium (IC_{50} value of 579 µg/mL) whereas ethyl acetate extract of culture filtrate reached IC_{50} value of 2,246 µg/mL. Table 1 presented the IC_{50} values of *A. auricula* extract.

Table1. IC_{50} values of *A. auricula* extract

No	Treatments	IC_{50} (µg/ml)
1	*A. auricula* ethyl acetate extract of the fruitbody	53
2	*A. auricula* ethyl acetate extract of the mycelium	579
3	*A. auricula* ethyl acetate extract of the culture filtrate	2,246
4	Doxorubicin	15,80

The linear regression analysis correlating the concentration of *A. auricula* extract to HeLa cell viability indicated the inhibition of HeLa cell growth. Ethyl acetate extract of fruitbody (Figure 1), and mycellium (Figure 2) demonstrated sharper decreasing slope than culture filtrate (Figure 3), i.e., 0.092, 0.099, and 0.02 respectively. The antiproliferative test revealed an increasing trend over time except for Doxorubicin (positive control) (Figure 4). Figure 5 represented the micrograph of HeLa morphological cells showing the damaged and healthy cells.

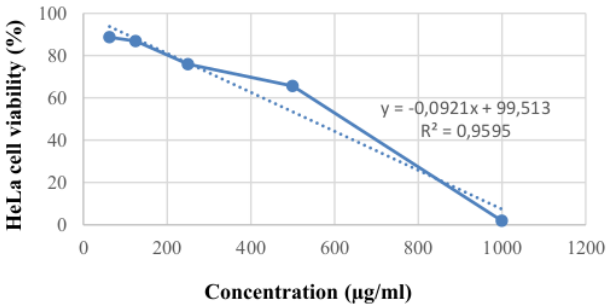

Figure 1. Linear regression curve for ethyl acetate extract of *A. auricula* fruit body

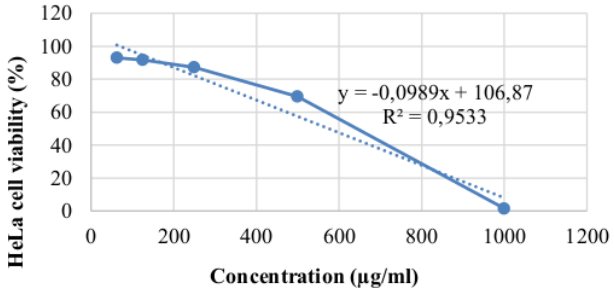

Figure 2. Linear regression curve for ethyl acetate extract of *A. auricula* mycelium

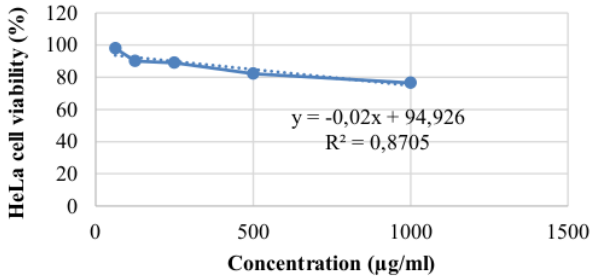

Figure 3. Linear regression curve for ethyl acetate extract of *A. auricula* culture filtrate

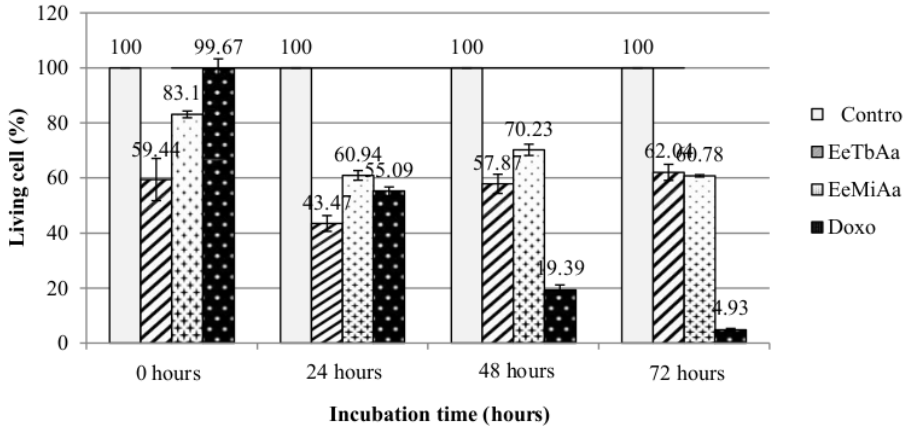

Figure 4. Antiproliferative test for ethyl acetate extract of *A. auricula* (EeTbAa: fruit body, EeMiAa: mycelium).

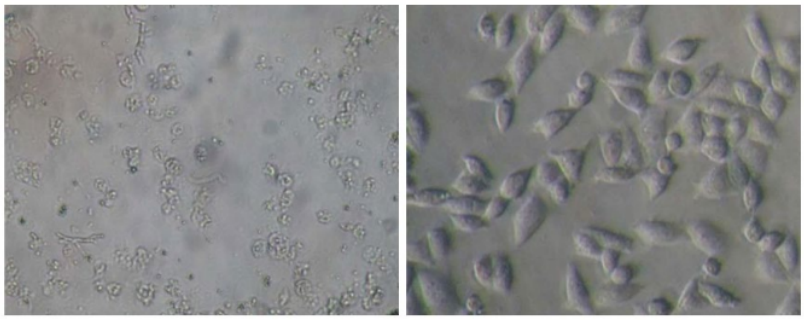

Figure 5. HeLa cell morphology from cytotoxic tests (left: damaged HeLa cells, right: healthy HeLa cells).

4. Discussion

The IC_{50} values indicate that the two types of extract (fruit body and mycelium) have potential anticancer compounds for HeLa cells. The ethyl acetate extract of culture filtrate showed value higher than 1,000 µg/mL suggesting no potential anticancer compound. An IC_{50} value of 45.3 µg/mL from ethyl acetate extract of *A. auricula* fruit body cultivated in Egypt was able to inhibit HCT116 colon cancer cells [10]. Testing of ethyl acetate extract carried out in Korea reported that ethyl acetate extract (IC50 of 133 µg/mL) inhibited Sarcoma 180 cells [11]. The IC_{50} value $\leq$ 1000 µg/mL suggests a good anticancer activity and that the extract is potential for further testing [12].

The fruit body and mycelium extract of *A. auricula* showed to inhibit HeLa cell viability. The viability of Hela cells decreased sharply as the concentration of *A. auricula* extract increased. At the extract concentration of 1,000 µg/ml, the HeLa cell viability reached a zero value indicating death. This differed from culture filtrate extract that demonstrated a slow decrease of HeLa cell viability. This condition was possibly due to the capacity of bioactive compounds in the extract was low, thus unable to suppress HeLa cell growth. Even in a concentration of 1,000 µg/ml the extract was not able to suppress HeLa cell growth by 50%. Thus, the extract of culture filtrate is not potential as a HeLa cell anticancer agent.

The subsequent tests were antiproliferative test and apoptosis test. The tests were only used for extracts of fruit body and mycelium of *A. auricula*. The fruit body extract of *A. auricula* mushroom has been studied and claimed that *A. auricula* contains alkaloid compounds, flavonoids, saponins, steroids, and carbohydrates [13]. With the various contents of the bioactive compounds, *A. auricula* is very potential as an anticancer compound.

Observation of apoptosis test on HeLa cells showed that the ethyl acetate extract of A. auricula fruit body and mycelium induced apoptosis. Red marks of the DNA showed the apoptosis as a result of ethidium bromide dye penetrating the cells through the damaged membrane. The apoptosis was characterized by fragmented, damaged cells. There were visible circles in cells that were still covered by cell membranes. A small number of cells also experienced necrosis characterized by the cell membrane rupture so that the cell cytoplasm spilled out and toxic for the surrounding cells. *A. auricula* extract might damage cancer cells through chromatin disintegration mechanism that induces apoptosis [14].

5. Conclusion

The results showed that the best cytotoxic effect of Jew's Ear Mushroom extract was the mushroom fruit body with an IC_{50} value of 538 µg/ml (medium level). Antiproliferative analysis toward HeLa cells showed the best inhibitory effect in ethyl acetate extract of mushroom fruit body and the best part-time effect was at 24-hour incubation. The apoptosis test showed that cell death through apoptosis mechanisms was more than necrosis.

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