Ethanolic Extract of Moringa's leaves (Moringa oleifera) Induce Senescence on Adenocarcinomic Alveolar Basal Epithelial Cells (A549 Cell-lines)

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***Abstract*—Moringa (Moringa oleifera) is a plant that widely used as traditional medicines. The use of Moringa as herbal medicine until now is widely studied due to its high antioxidant activity and its role as an anticancer in vitro. This study aims to determine the effect of the ethanolic extract of Moringa's leaf on the viability and senescence of lung cancer cells (A549 cell-lines). Moringa leaves were extracted with 70% ethanol solvent and tested for cell viability by MTS test. Senescence detection test were conducted by Senescence Associated-β-Galactosidase (SA-β-Gal) staining. The result of the viability test showed that the ethanolic extract of Moringa leaves was able to reduce the viability of cancer cells by 1000 µg / ml of IC50 value. The senescence detection test results used SA-β-Gal showed that the ethanolic extract of Moringa leaves was able to induce senescence up to 25% in cancer cells. The same test also performed on normal cells (MCF-12A cell-lines). The result of the assay that conducted on normal cells showed that the ethanolic extract of Moringa leaves conduct greater toxicity in cancer cells, and did not significantly induce senescence in normal cells. These results indicate the potential of Moringa as a cancer therapy agent and could be used as a basis for further research on the potential for Moringa as an anticancer.**

Keywords—Anticancer, ethanolic extract of Moringa, in vitro, senescence, viability

I. Introduction

People has long been used plants as medicinal sources. Some plants that commonly used as herbal medicines are noni (Morinda citrifolia), ginger (Zingiber officinale) and moringa (Moringa oleifera).

Moringa is part of Moringaceae family, and a plant native to the Middle East (including India, Pakistan, and Bangladesh) and has been known as one of Ayurveda's medicines [1] [2]. The phytochemical study of moringa shows that moringa contains flavonoids, and glucosinolates which is the precursor of isothiocyanates formation. Both of these compounds have been long studied due to its high antioxidants activities as well as its potential as anticancer [3].

Cancer is a disease characterized by uncontrollable cells growth and spreads also is the second leading cause of death in the world. Global-scale cancer estimates that one in five men and one in six women with cancer, and one in eight men and one in 11 women die from cancer. Among the various types of cancer, lung cancer is the most common cancer case as well as the leading cause of death from cancer [4].

Due to its high number of case and death from cancer, many studies for anticancer agents has been studied. Several targeted mechanisms of anticancer in in vitro studies against cancer including induction of apoptosis, induction of reactive oxygen species (ROS), and induction of cells senescence [5][6][7].

Induction of senescence is one of the targeted mechanism in cancer treatment. The phenomenon of senescence first described in 1961, when Hayflick and Moorhead [8] conduct human fibroblast cells cultured in vitro. Hayflick and Moorhead [8] found that in vitro cells culture has a limited division, which then known as Hayflick limit. Hayflick Hypothesis [9] states that "The limited time of cell strain in vitro possibly due to ageing expression or senescence in cellular level". This limited-time of cells proliferation is closely related to a structure named telomeres [10].

Senescence is one of the mechanisms of tumour suppressor. Transformation of the normal cells into cancer leading cells to lose its senescence marker [10]. Result of the previous study shows that cancer could be forced to undergo senescence, thus senescence becomes one of the target mechanism in reserch against cancer.

Studies for potential anticancer agents continues to develop. The previous study on the potential of moringa as anticancer shows that soluble extract of moringa inhibits the growth of lung cancer [11]. The different types of solvents in extraction could affect the compound contents of the taken samples. Extraction with ethanol solvent has the highest polyphenol content [12]. Therefore, the research was carried out on the effect of Moringa leaf ethanol extract on A549-cell lines.

II. METHOD

A. Samples collection

Moringa leaf was collected from Ciwaregu, Bandung-Indonesia. The obtained moringa next documented and separated between leaf and the other organs.

B. Moringa Leaf Extraction

The method used for moringa leaf extraction was maceration method. Fresh moringa leaf was dried using air-dry and prevented from direct sunlight. Dried moringa leaf then grinned and filtered (100 mesh). Moringa leaf powder soaked in 70% distilled ethanol. The filtrates then concentrated using rotary evaporimeter to obtain the paste-shaped extract. Freeze-dry method then used to obtain the powder-shaped extract.

C. Determination of Concentration

The final concentration that used in this study was determined from IC50 value obtained from cells viability test result. The concentration series used for cells viability assay was 31,25; 62,5; 125; 250; 500; 750, and 1000 µg/ml. This concentration series is a modified series from a study of Moringa oleifera by Al-asmari et al [13], namely 500, 250, 0 µg/ml. The concentration series of this study obtained by making a working solution. The working solution is made by dissolving 1000 7500, 500, 2500, 1500, 625, and 321,5 µg/ml in 1 ml DMSO 10%.

D. A549 and MCF-12A Cell-lines culture

A549 cell-lines obtained from Biomolecular and Biomedical Center, PT Aretha Medika Utama, Bandung-Indonesia. Cell-lines were cultured in the complete medium F-12K and added with 10% FBS (Fetal Bovine Serum), 1% ABAM, 1% nanomite, and 1% Ampβ. Cell-lines are removed from cryo tank containing liquid nitrogen (-196˚C) and then put in a water bath that has been filled with water at a temperature of 370C until it melts. The cell then inserted into a falcon tube with 9 ml of culture medium. 1% ABAM, 1% nanomite, and 1% Ampβ), then centrifuged at 1600 rpm for 5 minutes. The supernatant then removed and the pellet is resuspended with 5 ml of culture medium and transferred to a sterile 25 cm2 tissue culture flask (T-flask). The cells were then incubated in an incubator at 370C, 5% CO2.

E. Cells viability Assay (MTS Assay)

Cells were plated in 96 well-plates with 180 µl growth medium, then incubated for 24 hours under conditions (37oC, 5% CO2). The medium then replaced and 10 µl working solution was added and re-incubated for 24 hours. A total of 10 µl of MTS reagent was added to each well. The cell is then incubated for 3 hours. MTS test results are known by using a spectrophotometer at 490nm OD [14].

F. Senescence detection assay (Senescence Associated-β-Galactosidase)

Cells were plated in 96 well-plates with 180 µl growth medium, then incubated for 24 hours under conditions (37oC, 5% CO2). The medium then replaced and 10 µl working solution was added and re-incubated for 24 hours. A total of 10 µl of MTS reagent was added to each well. The cell is then incubated for 3 hours. MTS test results are known by using a spectrophotometer at 490nm OD [14].

G. Senescence detection assay (Senescence Associated-β-Galactosidase)

Cells were plated on 24 well plates with 900 µl medium and incubated for 24H. A total of 100 µl working solutions and controls were added to the well. The concentration series used in the senescence detection assay was obtained from the IC50 value of the cell viability test. Cells that had been treated with Moringa leaf extract on 24 well-plates were incubated for 24 hours. The cell medium is then discarded, and the cell is washed with PBS solution 1x. Cells were fixed with 2% formaldehyde / 0.2% glutaraldehyde for 5 minutes, then washed again with 1x PBS solution. The SA-β-Gal dye was then added to each well and cells were incubated under conditions of 37ºC overnight. Cell staining results are seen through a light microscope [15].

III. RESULT

The results of the cells viability test (A459 cell-lines) show a tendency in decreasing of cells viability as the increase of treatment concentrations. The higher of the ethanolic extract of moringa was used, the fewer cells that viable. The highest concentration (1000 µg/ml) shows ±60% viable cells in lung cancer cells and go higher with the decrease of concentration until it reached ±90% of viable cells in the lowest concentration (31,25 µg/ml). In normal cells (MCF-12A cell-lines) 1000 µg/ml concentration shows ±70% viable cells and 31,25 µg/ml concentration shows ±90% viable cells (Figure 1.)

The results of the viability test on both cells showed that the ethanolic extract of Moringa leaf reduced the viability of the cells to a greater number of cancer cells compared to normal cells. following the results of Jung's research (2014) regarding the effect of water extracts of Moringa leaves on lung cancer cells that Moringa leaf extract shows a greater cytotoxicity value on cancer cells compared with normal cells. Moringa leaf water extract with a concentration above 300 µg / ml can reduce the viability and proliferation of lung cancer cells (A549) up to 50% with a treatment period of 24 hours.

The comparison of cell viability test results from both cell types in normal control and 10% DMSO control did not show significant results. These results show that the 10% DMSO as extract solvent has no influence on the cell, so the research carried out avoids bias. Comparison between the treatment of ethanol extract of Moringa leaf within control showed significant results. The concentration of doxorubicin used (20 µg / ml) reduces the viability of cancer cells by ± 50%, and normal cells by ± 90%. This result shows that doxorubicin is more toxic in normal cells compared to cancer cells. Moringa leaf ethanol extract also has potential as an anticancer drug because it is safer than doxorubicin as indicated by the concentration of 1000 µg / ml extract not significantly different from doxorubicin control in cancer cells, but significantly different in normal cells. The results of this study are consistent with the results of the study of Al-Asmari et al [13], where the leaf and stem extracts from Moringa reduce viability in breast and colon cancer.

IC50 values for lung cancer cells (A549 cells) and normal cells (MCF-12A cells) were obtained by conducting a probit test. The IC50 value for cancer cells was 1062,878 µg / ml, while the IC50 value for normal cells was 1424,048. These results show that the ethanol extract of Moringa leaves is safer for normal cells compared to cancer cells.

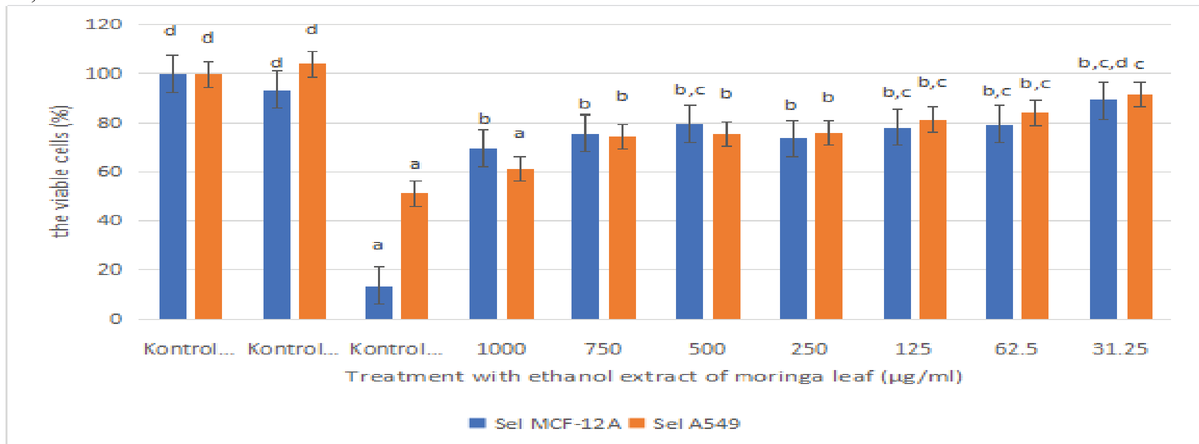

Figure 1. The viability value of lung cancer cells (A549 cells) and normal cells (MCF-12A cells). Data are presented in mean standard deviations. Different letters in the same type of cell show that there is a significant difference in α≤0.05 based on the Bonferroni test

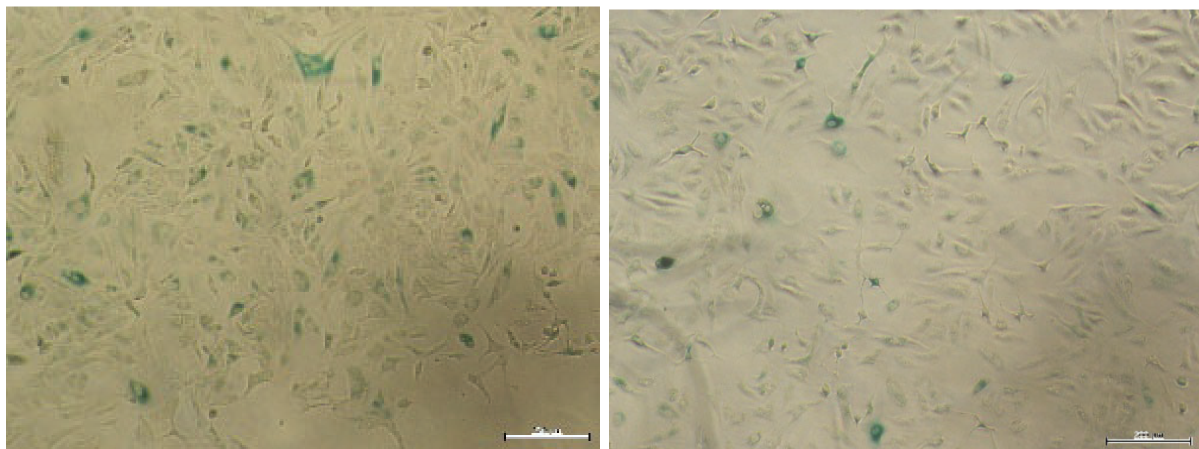

Figure 2. the result of senescence detection test using SA-β-Gal staining in lung cancer cells (A549). (a) extract concentration of 1000 µg / ml; (b) normal control (Personal documentation, 2019)

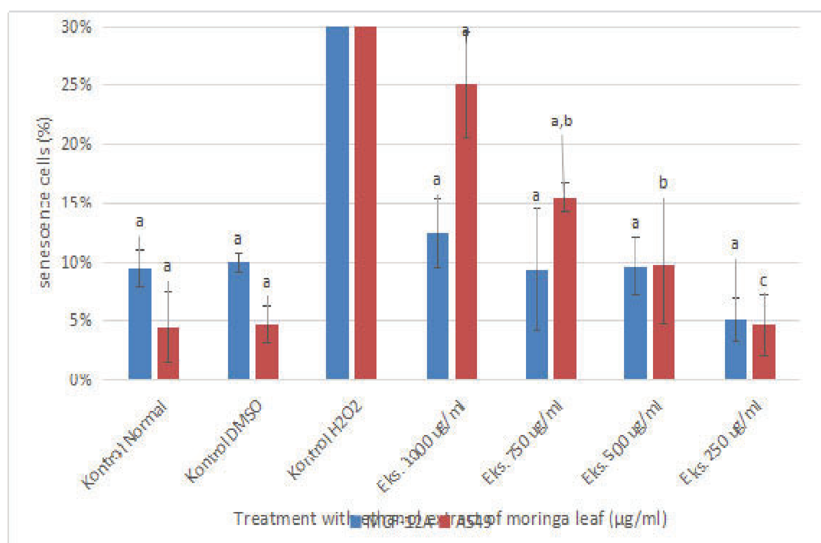

Figure 3. Results senescence detection of lung cancer cells (A549) and normal cells (MCF-12A) cells. Data are presented in mean standard deviations. Different letters in the same cell type show that there is a significant difference in α≤0.05 based on the One Way ANOVA test followed by the Tukey test)

A. *Senescence Cells Detections (SA-β-Gal Assay)*

The appearance of the result of cells staining by SA-β-Gal is shown in Figure 2. The green patches in Figure 2 are the senescence cells. The results of the quantification of senescence staining showed that the treatment of ethanol extract of Moringa leaves had different effects on cancer cells and normal cells (Figure 3). Moringa leaf ethanol extract induces senescence with a greater percentage in cancer cells than in normal cells. The lowest concentration of Moringa leaf ethanol extract (250 µg / ml) induces ± 5% senescence in lung cancer cells, and it increases with increasing concentrations, to reach ±25% of cells that senescence at the highest concentration (1000 µg / ml).

These results show that the ethanol extract of Moringa leaves was able to induce senescence in lung cancer cells. In normal cells, the lowest concentration of Moringa leaf ethanol extract (250 µg / ml) induces ± 5% senescence, while the.

The content of isothiocyanate (ITC) as a secondary metabolite in moringa leaves is thought to induce senescence in lung cancer cells (A549). The results of reviews conducted by Navarro et al [16] regarding the content of ITC in Moringa, stated that the content of ITC in Moringa acts as a chemoprotective agent in cells. The most abundant secondary metabolite compounds in Moringa are also able to induce cancer cells to enter a period of total rest (senescence). ITC acts as an anticancer by being involved in several signalling pathways, one of which is by forcing cancer cells into the senescence phase.

IV. CONCLUSION

Based on the results of research that has been done, it can be concluded that the ethanol extract of Moringa oleifera leaves reduces viability and induce senescence of lung cancer cells (A549 cells). Moringa leaf ethanol extract has greater senescence and toxicity induction value to cancer cells compared to normal cells.

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