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The Potential of *Moringa oleifera* Leaf Ethanolic Extract as Anticancer Against Lung adenocarcinoma (A549) Cells and Its Toxicity on Normal Mammary Cells (MCF-12A)

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Abstract

Moringa oleifera (MO) is traditional medicine with variety biological activities. *M. oleifera* as anticancer agent is due to its low toxicity to normal cells and high toxicity to cancer cells. This research aims was to evaluate the *M. oleifera* leaf extract (MOE) anticancer activities against lung adenocarcinoma cell lines (A549) and its toxicity on normal breast human cells (MCF-12A). The evaluation includes viability assay, intracellular ROS detection, apoptosis detection, and cells senescence detection assay. The viability assay was conducted using MTS cytotoxicity assay, and the senescence assay was carried out by Senescence Associated-β-Galactosidase (SA-β-Gal) staining. ROS and apoptosis detection assay were determined by flow cytometry using DCFDA staining for ROS detection and PI/Annexin-V staining for apoptosis assay. The results reveal that MOE have higher toxicity against A549 cell lines with IC_{50} 1062.87 µg/mL compared to MCF-12A cell lines with IC_{50} 1424.04 µg/mL. The MOE induces ROS levels up to 89.71±0.58 % in MOE 1000 µg/mL treatment. Furthermore, 15.32±0.33 % cells were late apoptosis and 48.69±0.10 % cells were necrosis after 24 h treatment by MOE 1000 µg/mL. The ability of MOE 1000 µg/mL to trigger senescence was higher in A549 cells (25.04±2.9 %) compared to MCF-12A cells (12.48±4.6). The results indicate that the MOE have higher toxicity against cancer cells compared than normal cells, and MOE can kill cancer cells through generating intracellular ROS levels, inducing apoptosis and triggering senescence in A549 cell lines.

Keywords: Anticancer, A549, MCF-12A, *M. oleifera*, ROS

Introduction

Cancer is one of the diseases suffered by many people in the world, and the incidences are projected to increase by around 70 % in the next 2 decades. Based on World Health Organization (WHO) data, cancer ranks the first or second cause of death before age 70 years at least in 91 countries world-wide [1]. Specify for lung cancer, this cancer is the deadliest cancers in the world, ranked first among men and second among women. Air pollution and smoking behavior is responsible to the increasing cases of lung cancer, and due to the both causes, the cases are projected to increase [2].

One of the factors is expensive anticancer drug and inadequate healthcare system. Affordable and inexpensive anticancer drug from medicinal plant seem to be the solution to overcome the problem [3]. Many medicinal plants have been used to treat cancer, and surprisingly, approximately 60 % of current anticancer drugs are acquired from natural sources such as medicinal plants [4].

Moringa oleifera is a native plant from India and classified into Moringaceae family. *M. oleifera* has been used as traditional medicine in many countries [5]. It also sources of valuable nutrients such as proteins, fats, vitamins, potassium, iron, and other nutrients [6,7]. Research reveal that *M. oleifera* has many biological activities, including antiviral [8], antioxidant [9], antimicrobial [10], anti-inflammatory [11,12], and anticancer [3].

The interest to use *M. oleifera* as anticancer has risen since it has lower toxicity to normal cells and high toxicity to cancer cells [13]. *M. oleifera* contains polyphenols and polyflavonoids that has antioxidant and potential anticancer activity [14]. Several compounds found in *M. oleifera* such as niazimicin, benzyl-isothiocyanate, 4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate have anticancer activities. Research have suggested that the compound that have anticancer activities is niazimicin [15].

Studies have tested and investigated anticancer activities of all part of *M. oleifera* plant in several cell lines. Jung *et al*. [16] stated that MO leaf extract induce apoptosis in human hepatocellular carcinoma HepG2 cells by inducing caspase and poly(ADP-ribose) polymerase cleavage. Al-Asmari *et al*. [17] revealed that ethanol extract of MO poses cytotoxic activity against ileocecal adenocarcinoma (HCT-8) and human breast adenocarcinoma (MDA-MB-231). The results also exhibited that leaf extracts have the highest anticancer activity compared to other parts of the plant. Other research also reveal the anticancer potential of MO leaf extract against various cell lines such as A549 (lung adenocarcinoma) [18], Hep-2 (HeLa contaminant carcinoma) [19], IMR-32 (neuroblastoma) [20], U266B1 (B-lymphocyte plasmacytoma) [21], Caco-2 (colorectal carcinoma) [22], and MCF-7 (breast adenocarcinoma) [22].

There are many pathways in cancer cell cytotoxicity by natural products. Natural compound found in plants can induce apoptosis, senescence, and regulate several genes that responsible in cancer suppression pathways such as NF-kB, Nrf2 pathway, and Wnt pathway [23]. Apoptosis is the most common pathway in cancer suppression by natural products [24]. To explore more in depth about the mechanism of *M. oleifera* leaves ethanolic extract against cancer cells, we examined senescence and apoptosis assay and also measured the reactive oxygen species (ROS) levels in A549 cell lines.

Materials and methods

MO leaves was collected from Ciwaregu region (Bandung, Indonesia). A549 cells and MCF-12A cell were obtained from Aretha Medika Utama, Ltd (Bandung, Indonesia). Senescence assay kit were purchased from Abcam (ab65351). MTS cell viability assay was purchased from Abcam (ab197010). ROS assay kit was purchased from Abcam (ab113851) and PI/Annexin-V cell apoptotic assay kit was purchased from BioLegend (79998).

MO leaf extraction

The MO leaf was dried through air-dry and prevented from direct sun light. The dried leaf was then grilled and sieved to obtain 100 mesh dried leaf. The simplicia of MO leaf which was put into a macerator weighing 120 g was dissolved with 500 mL ethanol at first day. The leaf powder was then extracted through overnight maceration method in 70 % ethanol. The time required for maceration is 3 days from a total of 120 g of MO leaf powder and a total of 1500 mL ethanol solvent to produce 1388 mL of filtrate. The filtrate faced to evaporation in rotary evaporation at 82 - 100 °C. The powder-shaped extract was then gained through freeze-drying method.

A549 and MCF-12A cell culture

A549 cell line was cultured (5 % CO_2, 36 °C) in 25 and 75 cm^3 disposable flask in complete F12K medium added with 10 % FBS (Biowest, S1810-5000), 1 % ABAM (Biowest, L0010-100), 1 % Nanomycopulitine (Biowest, LX16-100), 0.1 % Gentamicin (Gibco, 15750060), 1 % Amphotericine B (Biowest, L0009-050). MCF-12A cell line was cultured (5 % CO_2, 36 °C) in 25 and 75 cm^3 disposable flask in DMEM-F12K medium supplemented with 20 ng/mL human epidermal growth factor, 100 ng/mL cholera toxin, 0.01 mg/mL bovine insulin (Sigma Aldrich, I6634), 500 ng/mL hydrocortisone, 5 % horse serum, 1 % ABAM (Biowest, L0010-100), 1 % Nanomycopulitine (Biowest, LX16-100), 0.1 % Gentamicin (Gibco, 15750060), 1 % Amphotericine B (Biowest, L0009-050). The cells were incubated in an incubator at 37 °C, 5 % CO_2, medium changed twice a week until confluency.

Cells viability assay (MTS assay)

The cell viability was analyzed using MTS cell viability assay kit (Abcam, ab197010) under manufacturer's protocol. Cells were seeded in 5×10^3 in 96 well-plates with 200 µL F12K complete

medium with 2 % FBS, then incubated in an incubator (37 °C, 5% CO_2) for 24 h. The medium was then replaced and the cells were treated with MO leaf extract with 1000; 500; 250; 125; 625; 31.25 (µg/mL) final concentrations in 3 replicates, and were then re-incubated for 24 h. A total of 10 µl of MTS reagent was added to each well. The cell was then incubated for 3 h. MTS assay results are known by a spectrophotometer at 490 nm OD.

Senescence detection assay (senescence associated-β-galactosidase)

The senescence detection assay was analyzed through senescence associated-β-galactosidase assay kit (Abcam, ab65351). Cells were plated on 24 well plates with 1000 µL medium and incubated for 24 h. The cells were then treated with MO leaf extract 1000; 500; 250 (µg/mL). The concentration series used in the senescence detection assay was obtained from the IC_{50} value of the cells viability assay. The cell medium is then removed and the cell were washed with 1X PBS solution. Cells were fixed with fixation solution (2 % formaldehyde or 0.2 % glutaraldehyde) for 15 min at room temperature, then re-washed with 1X PBS solution. The solution containing staining solution, staining supplement, and 20 mg/mL X-gal in DMF was then added to each well and cells were incubated overnight in 37 °C, CO_2-free incubator. The microscopic image was then captured using inverted light microscope and the number of senescence cells were then manually calculated [25].

Reactive oxygen species (ROS) detection

ROS levels were determined using flow cytometry using DCFDA (Abcam, ab113851) under manufacturer protocols. The cells that were overnight treated with 1000, 500, and 250 (µg/mL) MO leaf extract. The cells were harvested and 250,000 cells were placed in FACs round tube. The cells were next suspended with 20 µM DCFDA in 1 % PBS. The cells then retreated with TBHP (positive control), 1000, 500 and 250 (µg/mL) MO leaf extract. Then, the tubes were incubated for 4 h (5 % CO_2, 36 ºC). The ROS levels were then detected and calculated using flow cytometry (MACSQuant Analyzer 10, Miltenyi Biotec).

Apoptotic assay

Apoptotic assay was analyzed using flow cytometry using PI apoptotic kits (BioLegend, 79998) under manufacturer protocols. The cells were plated in 6 well plates and incubated in an incubator (5 % CO_2, 36 °C) for 24 h. The cells were then induced with 1000, 500, and 250 (µg/mL) MO leaf extract and re-incubated for 24 h. After treatment, the cells were harvested and placed in FACs round tubes, the cells were washed in 500 µL Annexin Binding Buffer 1× twice. The cells were then suspended in 100 µL Annexin Binding Buffer 1× and stained with Annexin V-FITC and PI-Per Cp. Cy5 for 30 min in dark condition (37 °C, 5 % CO_2). The apoptotic profile was then analyzed using flow cytometry (MACSQuant Analyzer 10, Miltenyi Biotec).

Statistical analysis

The data were analyzed using SPSS ver 22.0 software. One-way analysis of variance (ANOVA) was used to analyzed, followed Tukey HSD post-hoc test and Bonferroni post-hoc test and $p < 0.05$ were considered significant.

Results and discussion

Figure 1 reveals the result of cell viability assay of A549 and MCF-12A cells after 24 h treatments in various concentration of MOE. The result exhibit that there was a significant decreasing in the number of viable A549 and MCF-12A cells after MOE treatments. The treatments showed the effect on cell viability in concentration dependent manner, the higher concentration, the lower viable cells percentage. Using Probit Test in IBM SPSS 22.0 software, the IC_{50} for A549 was 1062.87 µg/mL and the IC_{50} for MCF-12A was 1424.04 µg/mL. This indicated that MOE was more toxic for cancer cells compared to normal cells. Nevertheless, the IC_{50} of the MOE was very higher compared to the results of other studies.

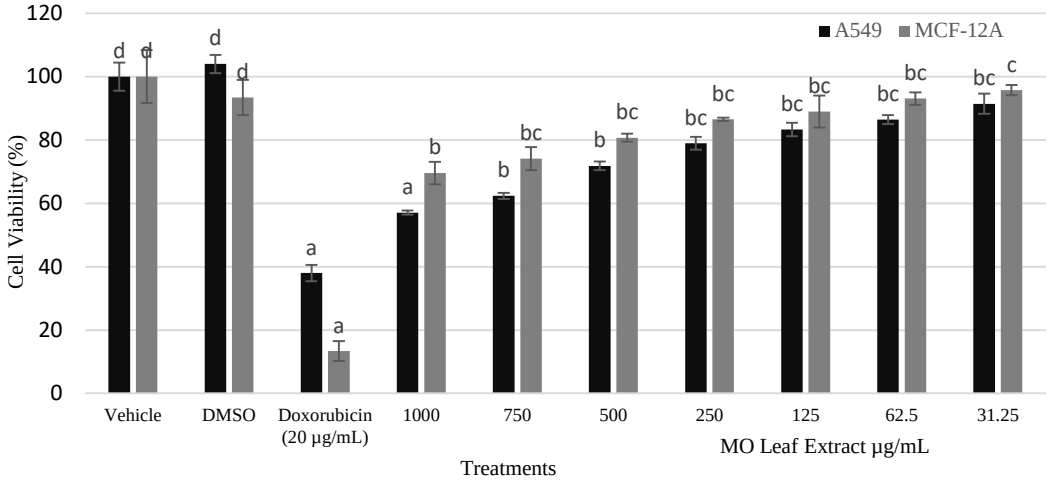

Figure 1 Cells viability of A549 and MCF-12A cells lines in various concentrations of MO leaf extract treatments. The different letters in the same cell lines (a, b, bc, c, d) were statistically significant in Tuckey post hoc test ($p < 0.05$).

Quantification of ROS levels reveal that the MOE treatment rise the ROS level significantly in A549 cell lines (**Figure 2**). 1000 µg/mL MOE give similar result with 20 mM TBHP, a compound that used as positive control. The level of intracellular ROS (%) were 13.51±4.15, 88.32±0.67, 89.71±0.58, 45.81±2.87 and 41.94±2.88 for vehicle, 50 mM TBHP, 1000 µg/mL MOE, 500 µg/mL MOE, and 250 µg/mL MOE, respectively.

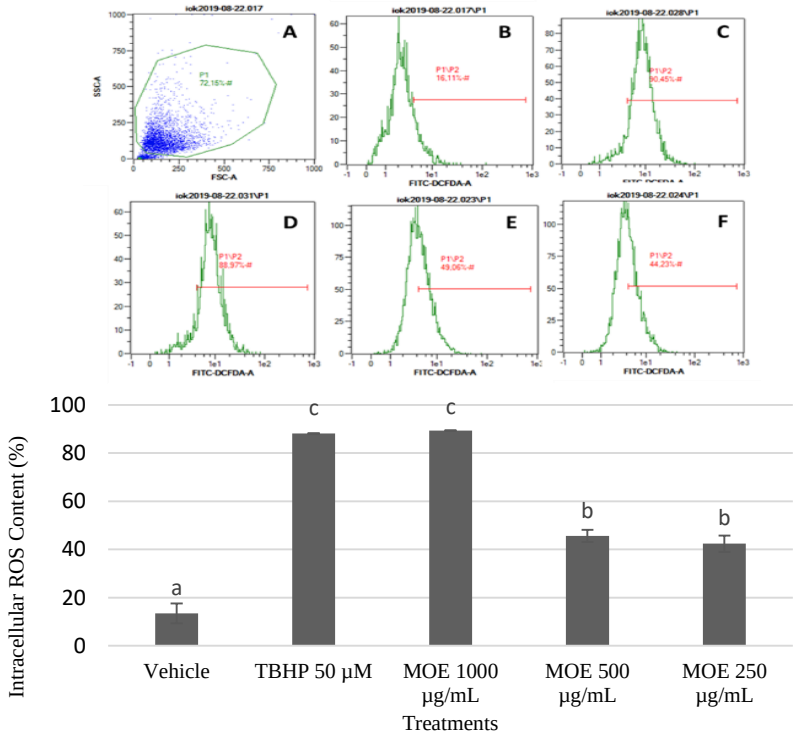

Figure 2 Intracellular reactive oxygen species levels in A549 cell lines after various treatment of MOE (upper), getting strategy (A), vehicle treatment (B), TBHP (C), MOE 1000 µg/mL (D), MOE 500 µg/mL (E), and MOE 250 µg/mL (F). The lower graph exhibits the number the percentage of ROS contents in various treatments. The different letters (a, b, c) were statistically significant in Bonferroni post hoc test ($p < 0.05$).

Dot blot of PI/Annexin V apoptosis assay detection of A549 cells in several MOE concentrations can be seen in **Figure 3**. The results reveal that MOE treatment increased the number of necrotic and late apoptotic cells significantly. The proportion of necrotic cells in vehicle group were 3.84±0.21 while the percentage for MOE 1000 µg/mL were 48.69±0.10. The percentage of late apoptosis cells for vehicle, MOE 1000 µg/mL, MOE 500 µg/mL, MOE 250 µg/mL treatment were 3.09±0.07, 15.32±0.33, 4.72±0.83, and 2.69±0.19, respectively. This result indicate that MOE extract induces necrotic and late apoptotic in A549 cell lines.

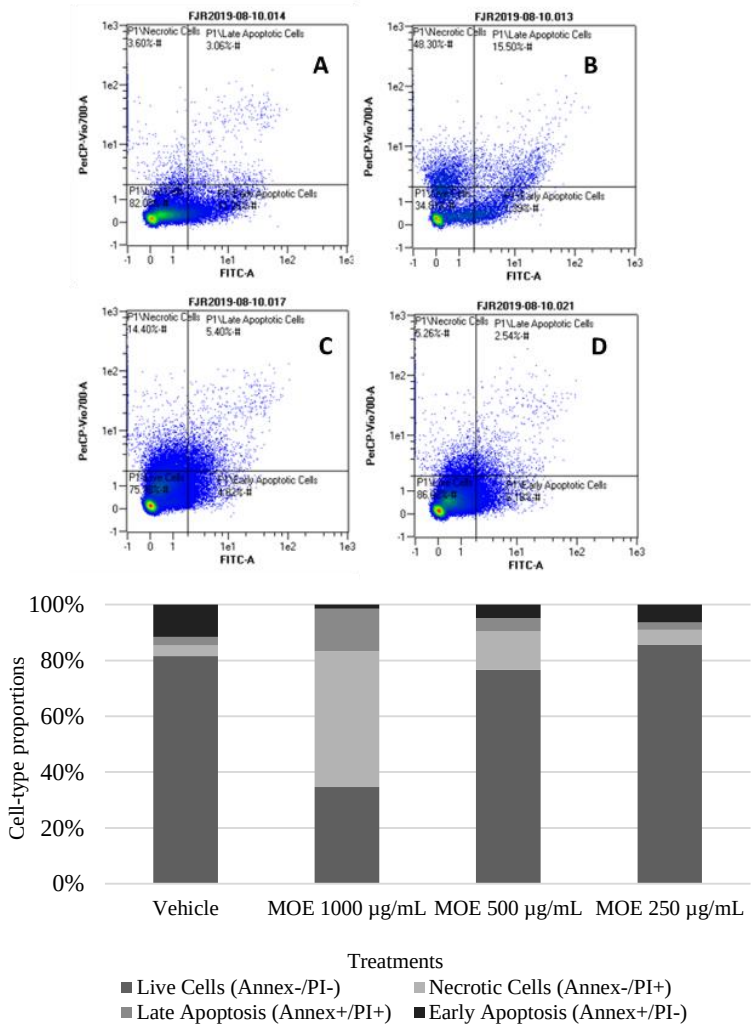

Figure 3 The dot blot of PI/Annexin V visualization by flow cytometric analysis. Upper figure A), B), C), and D) represent vehicle, MOE 1000 µg/mL, MOE 500 µg/mL, and MOE 250 µg/mL, respectively. The lower graph reveals the proportion of live, necrotic, early apoptosis, and late apoptosis cells proportion in A549 cell population after MOE treatment.

The visualization of senescence detection assay was revealed in **Figure 4**. MOE induces senescence in both normal cells and cancer cells. However, the proportion of senescence cells in cancer cells was greater than the percentage in normal cells. As comparation, the number of senescence in A549 treated with MOE 1000 µg/mL was 25.04±2.9 % and the figure for MCF-12A is 12.48±4.6 %. This phenomenon indicate that the ability of MOE to kill the cells through senescence pathway was higher in cancer cells compared to the normal cells. The lowest concentration of MOE (250 µg/mL) induces 4.68±1.85 and 5.11±2.54 % senescence in cancer and normal cells, respectively. The activity of MOE in senescence induction follows a concentration-dependent pattern.

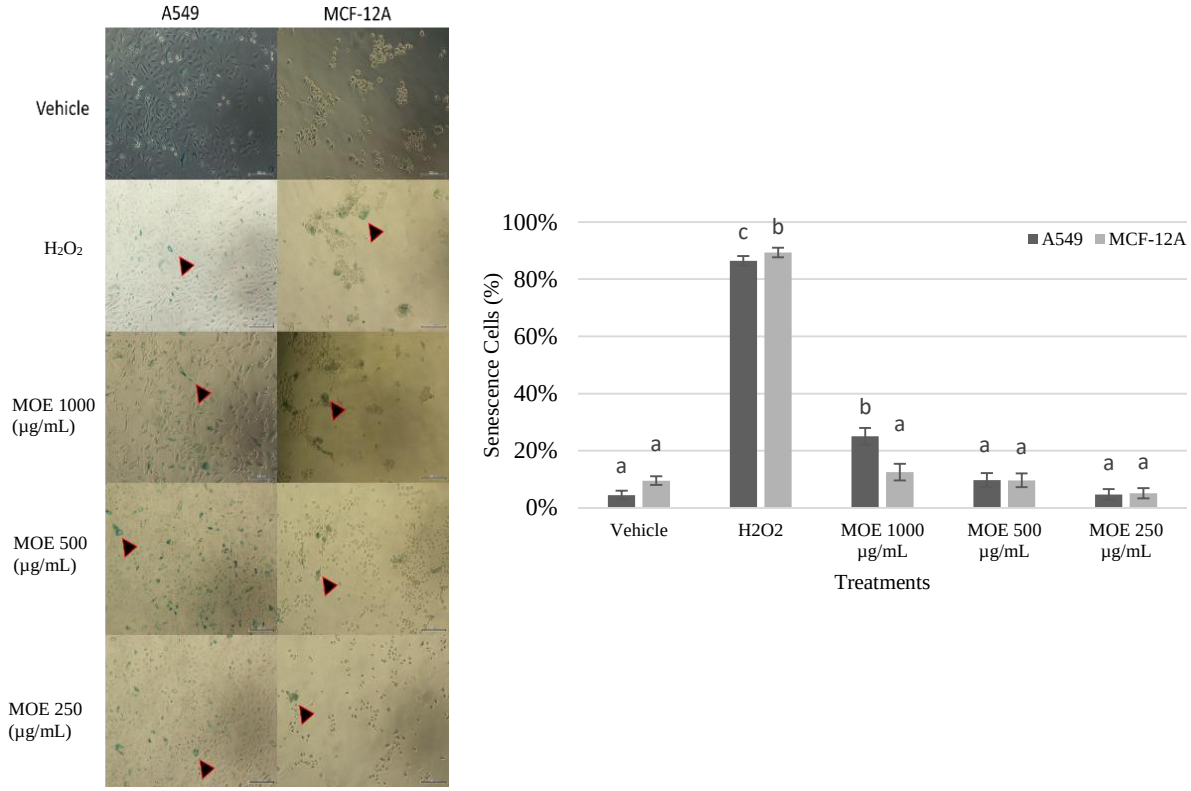

Figure 4 Visualization of senescence detection assay in A459 and MCF-12A cell lines. The cells with green color are senescence cells (black arrow). The bar chart exhibits the percentage of senescence cells. The different letters in the same cell lines (a, b, c) were statistically significant in Tuckey post hoc test ($p < 0.05$).

This research supports the previous research concerning the anticancer activities of *M. oleifera* and its toxicity in normal cells. *M. oleifera* poses anticancer activities against various cell lines and has low toxicity to normal human cells [3]. Compared to other studies, IC_{50} of MOE against A549 in this study was very high. Tiloke *et al.* [26] showed that the IC_{50} of MOE against A549 was 166.7 µg/mL and Jung [18] stated that almost 50 % cell die after 24 h treatment with *M. oleifera* water soluble extract. This research and both previous studies indicate that different samples, extraction methods, and assays give different result on IC_{50} value. The cytotoxicity of MOE in cancer cells was higher compared to the toxicity for normal cells.

Another study conducted by Abd-Rabou *et al.* [13] reveal the same finding, *M. oleifera* poses higher toxicity in HepG2, Coco-2, and MCF-7 (cancer cells, 80 %) rather than in BHK-21 (normal cells, 30 - 40 %)

Further, the number of ROS level in A549 was increase significantly after MOE treatment (**Figure 2**). In line with this result, *M. oleifera* extract rise lipid peroxidation by increased the concentration of MDA, and dropped the level of GSH. This was responsible for significant increase of lipid peroxidation and triggered oxidative stress of the cells [26]. The rise of oxidative stress was genotoxic to the cells. Free radicals such as H_2O_2 can react with metals in the biological system and produce highly reactive hydroxyl radicals that can cause DNA damage. ROS-mediated DNA damage is one of mechanisms of natural product against cancer cells as it can be signals that cause DNA strand breakdown [27]. Although he contradiction in the function of ROS in cancer cells, high ROS content in the cells in lethal level contributed to the rise of chemosensitization and the activation of several cell death pathways.

Next, MOE induces apoptosis and necrotic cells. The number of necrotic cells rise significantly from 3.84±0.21 % in vehicle treatment to 48.69±0.10 % in MOE 1000 µg/mL treatment. In addition, 15.32±0.33 % cells were late apoptosis in MOE 1000 µg/mL group compared to only 3.09±0.07 % cells

were late apoptosis in vehicle treatment. Proliferation inhibition through apoptosis is the main pathway of *M. oleifera* against cancer cells [28].

The p53 protein expression were highly regulate in DNA damage cells and promote DNA repair, if the DNA repair is unsuccessful, it then causes apoptosis and cell death [29]. MOE also increase the expression of Caspase-3 and Caspase 7/9, a protease that degrade cellular protein during cell apoptosis [30]. It is clear that the mechanism of MOE against cancer is due to its ability to elevate the intracellular ROS levels, trigger DNA damage, and induce the expression of various protein-related apoptosis such as p53, Caspase 7/9, and Caspase 3.

Moreover, the MOE also induces senescence in dose dependent manner. As stated before, 1000 µg/mL MOE induce 25.04±2.9 % senescence in A549 cancer cells. This result simillar with the study conducted by Hisam *et al.* [31]. in fibroblast cell lines. The cellular stress triggers upregulation of p53 in MOE treated A549 cells which then induces the cells into senescence.

The induction of ROS, apoptosis and senescence of A549 cells by MOE is caused by its natural compound components**.** The compound such as quercetin and kaempferol in MOE extract can trigger apoptosis, senescence, and downregulated protein expression that responsible for cells proliferation [32,33]. Phytochemical studies show that MOE has a high content of active compounds. One of them is phenolic compounds (caffeic acid, chlorogenic acid, ellagic acid, ferulic acid, gallic acid, and o-coumaric acid) and the ones close-related to MOE are gallic acid (±1034 mg/g dry weight), flavonoids (myricetin, quercetin, kaempferol, isorhamnetin, and rutin) with a total concentration varied between 5.059 - 12.16 mg/g dry weight [34]. Quercetin (47 %), kaempferol derivatives (30 %), and apigenin (20.9 %) are the most abundant types of flavonoids in Moringa leaves out of total flavonoids [35,36]. Bioinformatics study also exhibited that niazimicin, one of *M. oleifera* compound, has potential therapeutic drug against prostate cancer [15].

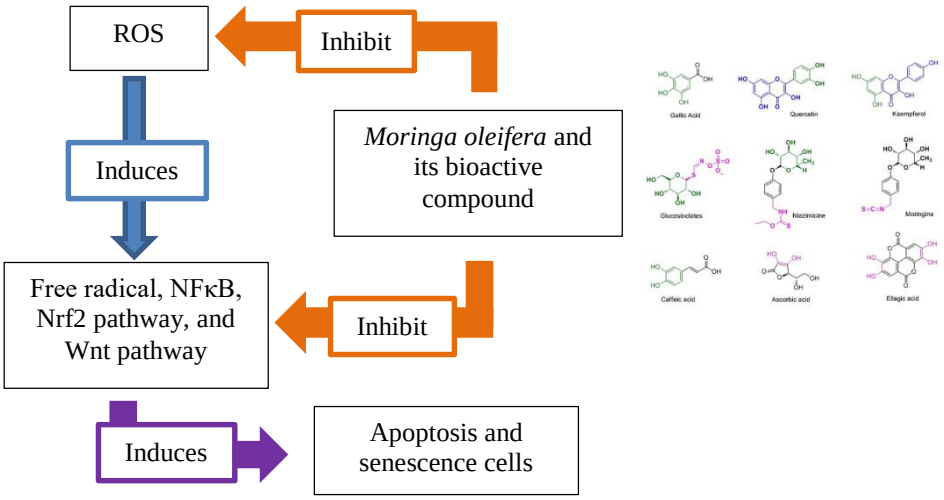

Figure 5 ROS induced damage and mechanism of action of *M. oleifera. M. oleifera* potential for induces apoptosis and senescence cells.

Conclusions

M. oleifera ethanolic leaf extract has antiproliferative ability against human epithelial alveolar lung cancer cells (A549) , but has lower toxicity on normal mammary luminal epithelial cells (MCF-12A) . MOE induces senescence, apoptosis, and necrosis in A549 cells by triggering cellular oxidative stress mechanism. Thus, the MOE has great potential as alternative anticancer treatment for lung cancer.

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