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Research article

Effect of ethanolic extract and n-hexane fraction of *Moringa oleifera* L. against cell cycle and apoptosis MCF-7 cell lines

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Key words: *Moringa oleifera*, Cell Cycle, Apoptosis, MCF-7 cell lines.

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Abstract

Objective: The aim of this study was to determine the effect of ethanolic extract (EE) and n-Hexane fraction (NHF) of *Moringa oleifera* L. leaf on cytotoxic effect, cell cycle and apoptosis on MCF-7 cell lines. **Methods**: In vitro Cytotoxic assays was determined by MTT (Microculture Tetrazolium Tehnique) assay, cell cycle inhibition and apoptosis were determined with flow cytometry. **Results**: Cytotoxic activity of EE with IC_{50} 94.44 µg / mL and 97.60 µg / mL value for NHF. The samples were caused cell accumulation in the G0-G1 phase and late necrotic region for apoptosis effect. **Conclusion**: EE and NHF are potentially to be developed as chemotherapeutic agent for breast cancer, while molecular mechanism needs to be explored.

Introduction

Chemotherapy drugs do not selectively kill cancer cells, but also cause normal cell death around cell lines that proliferate rapidly, such as cells in the hair follicles, gastrointestinal mucosa and bone marrow [1]. Cell lines is an accumulation of a number of genetic changes that play a role in the carcinogenesis, carcinoma progression and resistance to chemotherapy. Most of these genetic changes result in cell cycle regulation. In normal cells, there is a balance between cell proliferations with cell death regulated by the cell cycle with cellular checkpoint [2]. *Moringa oleifera* L. has been used as traditional medicine and food source in various parts of the world. Ethanolic extract of *Moringa oleifera* also has been proven to inhibit proliferation on Hela cancer cells [3]. Chemical properties of *Moringa oleifera* that has been reported to have anticancer activity are phenetyl isothiocyanate (PEITC) and benzyl isothiocyanate (BITC) [4-6]. Previous study reported isothiocyanate modified metabolic processes of carcinogenesis through inhibition of phase 1 metabolic enzymes and/or induction of phase 2 metabolic enzymes [7]. The flavonoid content found in Moringa leaves has the potential as an anticancer agent by inhibiting proliferation and inducing the apoptotic process of cancer cells [8]. Methanol extraction of Moringa leaf using Design of Experiment (DoE) software with response surface methodology in terms of temperature and incubation time by sonication obtained 88.39% cancer cell growth inhibition by this condition. Extracted leaves inhibited MCF-7 cell line with 87.13% in average at wavelength A570nm. [9]. Effect of *Moringa oleifera* leaf inhibiting the growth of MCF-7 cell lines has been proven, but effect of the active fraction of ethanolic extract of Moringa leaf on cycle cell and apoptosis on MCf-7 cell lines have never been done. Because of that, it is necessary to conduct research on the cytotoxic effects of ethanolic extracts and its active fraction on MCF-7 cell lines and its effects on cycle cell and apoptosis.

Materials and methods

Plant and chemicals materials

Fresh leaves of *Moringa oleifera* L. were collected from Bukit Batrem Village, East Dumai District, Riau Province, Indonesia. *Moringa oleifera* L. was identified in Herbarium Medanense (MEDA) University of Sumatera Utara no. 1786/MEDA/2017. Chemicals used were Doxorubicin (Ebewe), DMSO (Sigma), [3- (4,5-dimethylthiazole-2-yl) -2,5-diphenyl tetrazolium bromide] (MTT) (Sigma), Propidium Iodide kit (Biolegend), Annexin V (Biolegend).

Preparation of ethanolic extract (EAE) and n-hexane fraction (NHF)

An amount of 400 gram dried material plant samples were crushed in a blender, and then macerated in ethanol 96 % for 3 hours thereafter moved to percolator tube. Percolation was stopped if the last of 500 grams of solvent were evaporated, living no residuals. The solvent was evaporated at low pressure with a temperature of not more than 40º C using a rotary evaporator. It is then taken 20 g for liquid-liquid extraction to obtain a non polar using n-Hexane This study use ethanol and n-Hexane as

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solvent due to its characteristic as a polar and non-polar solvent which can pull out polar and non polar compound.

Phytochemical screening of ethanolic extract and n-hexane fraction

Phytochemical screening carried out on ethanol extract and n-Hexane fraction leaves of *Moringa oleifera* L includes examining the chemical secondary metabolites of alkaloids, flavonoids, glycosides, tannins, saponins, triterpenoids, and steroids.

Dosage of extract and doxorubicin

The treatment of extract used several concentration series of 1000 µg / mL; 500 µg / mL; 250 µg / mL; 125 µg / mL; 62.5 µg / mL; 31.25 µg / mL and 15.625 µg / mL. The treatment of doxorubicin used several concentration series of 24.00 µg / mL, 12.00 µg / mL; 6.00 µg / mL; 3.00 µg / mL; 1.50 µg / mL; 0.75 µg / mL and 0.375 µg / mL.

Cytotoxicity and selectivity index

MCF-7 cells and Vero cells used are from the Department of Parasitology Medical Faculty of Gadjah Mada University Yogyakarta. MCF-7 cells were grown on DMEM media and Vero cells with M199 media supplemented with 10% (Gibco) Fetal bovine, Penicillin 1% Streptomycin 1% (Gibco) and Fungizone 0.5% (Gibco) were incubated at 37°C, CO_2 5%. The inoculums seeded on a 96 well plate (Iwaki), each well 1 x 104 cells/0.1 mL. Cell cultures were incubated at 37°C, 5% CO2 for 24 hours. After 24 hours the media was discarded and the cell plus EE, NHF and doxorubicin were incubated for 24 hours then the medium was removed and 0.5 mg / mL of MTT was added and incubated for 4 hours at 37°C, 5% CO2. After crystal formazan was formed and 10% SDS was added to dissolve the formazan crystals, then incubated for 24 hours at room temperature and shielded from light. The absorbance was measured with microplate reader at λ 595 nm [10]. The resulting absorbance was converted to a percentage of cell viability, then the selectivity index (IS) EE and NHF were determined against MCF-7 cells [11-12].

The equation to determine the viability of cells

$$\%\text{Viability} = \frac{\text{Absorbance of treatment} - \text{absorbance of medium} \quad \text{x } 100\ \%}{\text{Absorbance of control cells} - \text{absorbance of medium}}$$

The equation to determine selectivity index (SI)

$$SI = \frac{IC_{50} \text{ on Vero cells}}{IC_{50} \text{ on MCF7 cells}}$$

Cell cycle inhibition assay

MCF-7 cells ($5x10^5$ cells/mL) were seeded into 6-well plate and incubated for 24 hours then treated with EE, NHF and doxorubicin, then incubated for 24 hours. After 24 hours the media was moved into the conical tube and then into well plus trypsin 0.025%, then washed with 2x PBS, collected into the conical and centrifuged at 2500 rpm for 5 minutes. The supernatant was thrown away, in the pellet added cold ethanol 70% for 2 hours for cell fixation. Then added with PBS, centrifuged at 3000 rpm for 3 min, the supernatant was removed, in pellets was added PI kit (containing 40 µg /g/µmL PI and RNAse 100 mL) and resuspended. Then mixture was incubated at 37°C for 30 minutes. The sample was analyzed by FAC Scan Flow cytometer. Based on its DNA content the percentage of cell accumulation in the cell cycle (G1, S, and G2/M) were calculated using ModFit Lt.3.0 [13].

Apoptosis assay

MCF-7 cells ($5x10^5$ cells/mL) were seeded into 6-well plate and incubated for 24 hours then treated with EE, NHF and doxorubicin, then incubating for 24 hours. After 24 hours the media is fed into the conical tube and then into well plus trypsin 0.025%. Then washed with PBS, collected into the conical and centrifuged at 2500 rpm for 5 minutes. The supernatant was thrown away, in pellets added PBS, the suspension was centrifuged at 3000 rpm for 3 min, the supernatant was removed and Annexin V kit added to the pellet and resuspended then incubated at 37°C for 30 minutes. The Sample was analyzed by FAC Scan Flow cytometer [5, 7].

Statistic analysis

Analysis of the cytotoxic test data was done using SPSS 24 software by probit analysis.

Result and discussion

Extraction of *Moringa oleifera* L leaves

The extraction of 400 grams of Moringa dried leaf obtained 74.44 grams of thick extract and stored at 2-8ºC.

Phytochemical screening result of that EE and NHF

Phytochemical screening results obtained can be seen in table 1.

Table 1. Result Phytochemical screening.

No	Screening	Ethanolic extract	n-Hexane fraction
1	Alkaloid	+	-
2	Flavonoid	+	-
3	Glycosides	+	-
4	Saponins	+	-
5	Antraquinone	-	-
6	Tannin	-	-
7	Triterpenoids/Steroids	+	+

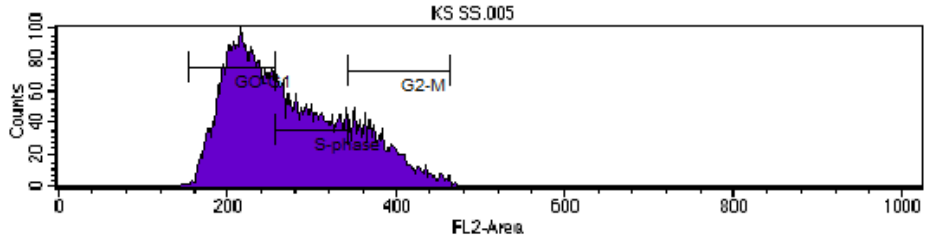
(+) = positively contain; (-) = negatively contain.

Inhibitory concentration 50% (IC_{50})

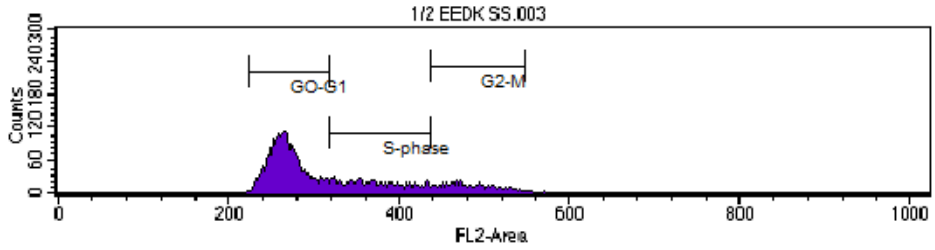
The cytotoxic effects of EE, NHF and doxorubicin against MCF-7 cells and their selectivity with Vero cells were determined by the MTT assay. In each EE and NHF treatment, doxorubicin was showed cell growth inhibition as indicated by IC_{50} values. The IC_{50} value of EE 94.44 µg/mL, NHF 97.60 µg/mL, doxorubicin 5.80 µg/mL. The selectivity of EE and NHF were determined with an executed cell viability assay on Vero cells. The treatment of EE and NHF were showed cytotoxicity effect on Vero cells with IC_{50} 373.29 µg/mL and IC_{50} 280.20 µg/mL for NHF. We compared IC_{50} of EE and NHF on Vero cells to MCF-7 cells to determine selectivity index (SI). Selectivity index of EE was showed 3.95 and SI of NHF was showed 2.87. SI>3 is supposed to be selective to MCF-7 cell lines. The result was showed that EE is more selective to MCF-7 cells instead of Vero cells than NHF [14].

Effect on cell cycle

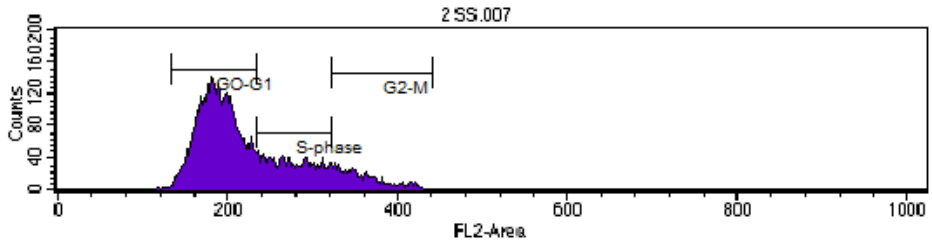
Determination of EE, NHF and doxorubicin in inhibiting cell cycle was done using flow cytometric method [13]. The effect of EE, NHF and Doxorubicin are given in Figure 1, whereas with treatment of doxorubicin at 2.90 µg/mL caused cell accumulation at G0/G1 phase (68.48%), EE at 47.22 µg/mL and 9.46 µg/mL caused cell accumulation at G0/G1 phase (63.52%) and (68.80%), NHF treatment G0/G1 cell accumulation (62.698%) and (69.88%) at concentration 48.80 µg/mL and 9.76 µg/mL. These results indicate that EE and NHF may increase the cytotoxic effect in the G0/G1 phase. Inhibition of the cell cycle by EE and NHF are probably caused by the effect of the active content contained in the leaves of *Moringa oleifera* L. that can inhibit the activation of Nuclear factor-kappa B (NF-kB). Which is NF-kB is a transcription factor that has an important role in cell growth and death cell, and development. Cell cycle regulatory barriers on G0/G1 phase by EE, NHF and doxorubicin occurs through a decrease in the level of expression of cyclin D1 so there caused activation of CDK4 and CDK6 which resulted in inhibition of phosphorylation pRb (retinoblastoma protein), which are not phosphorylated pRb will bound to E2F transcription factors bind to DNA and inhibit transcription of the genes whose products are required for S phase of the cell cycle, its caused G1 arrest [15-17].



a



b



c

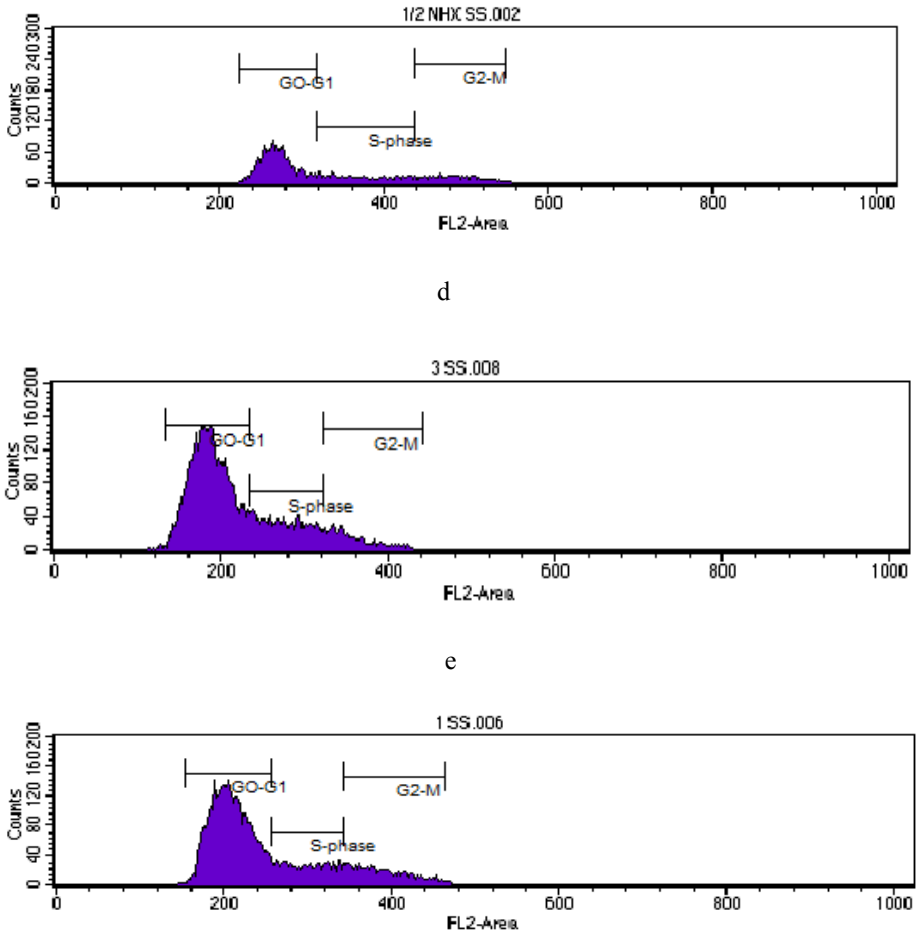
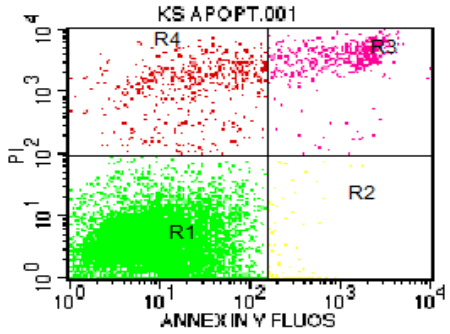


Figure. 1. Cell cycle analysis of EE, NHF and doxorubicin on MCF-7 cell lines. (a) control cells; (b) EE ½ IC_{50} (47.22 µg/mL); (c) EE $^1/_{10}$ IC_{50} (9.44 µg/mL); (d) NHF ½ IC_{50} (48.80 µg/mL); (e) NHF $^1/_{10}$ IC_{50} (9.76 µg/mL); (f) Doxorubicin ½ IC_{50} (2.9 µg/mL).

Effect on apoptosis

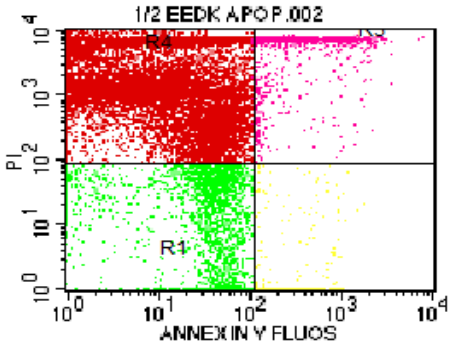
Determination of apoptotic induction was performed using the flow cytometry with addition Annexin V as shown in figure 2. Percentage control, EE ½ IC_{50}, EE $^1/_{10}$ IC_{50}, NHF ½ IC_{50}, NHF $^1/_{10}$ IC_{50} and doxorubicin ½ IC_{50} for early apoptotic 0.71%, 2.93%, 3.74%, 0.07%, 3.65% and 0.07%; in late apoptosis/early necrotic 3.51%, 5.89%, 3.49%, 10.02%, 10.08% and 8.62%; and in late necrotic 3.45%, 75.00%, 8.39%, 86.11% 62.57% and 88.90%. Apoptosis is the process of cell death programmed with cell morphological changes [17]. EE, NHF and doxorubicin may not induce apoptosis possibly due to MCF-7 cell lines had not showed *caspase 3* expression. Thus, caspase 3 expression of MCF-7 cell lines is an important role in inhibition of apoptotic pathway [18].



File: KS APOPT.001
Patient ID: 0424.18
Acquisition Date: 24-Apr-18
Gate: No Gate
Total Events: 15030

Region	% Gated	% Total
R1	92.36	92.36
R2	0.71	0.71
R3	3.51	3.51
R4	3.45	3.45

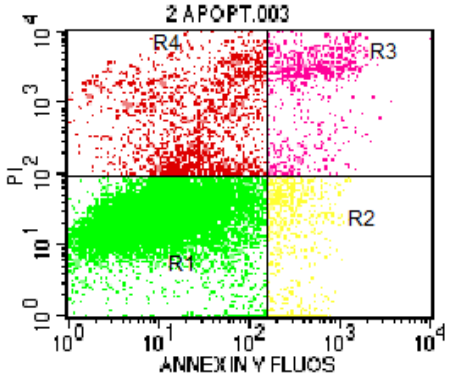
a



File: 1/2 EEDK APOP.002
Patient ID: 0413.18
Acquisition Date: 13-Apr-18
Gate: No Gate
Total Events: 20000

Region	% Gated	% Total
R1	16.36	16.36
R2	2.93	2.93
R3	5.89	5.89
R4	75.00	75.00

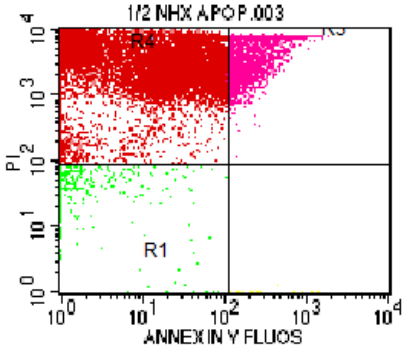
b



File: 2 APOPT.003
Patient ID: 0424.18
Acquisition Date: 24-Apr-18
Gate: No Gate
Total Events: 20000

Region	% Gated	% Total
R1	84.52	84.52
R2	3.74	3.74
R3	3.49	3.49
R4	8.39	8.39

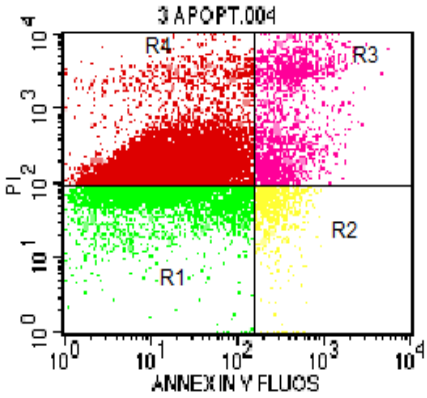
c



File: 1/2 NHX APOP.003
Patient ID: 0413.18
Acquisition Date: 13-Apr-18
Gate: No Gate
Total Events: 20000

Region	% Gated	% Total
R1	3.90	3.90
R2	0.07	0.07
R3	10.02	10.02
R4	86.11	86.11

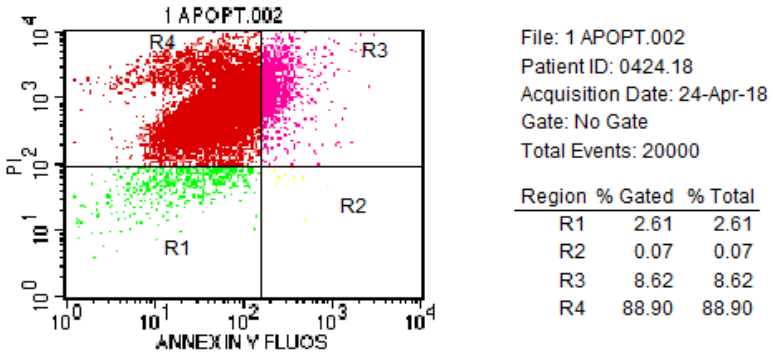
d



File: 3 APOPT.004
Patient ID: 0424.18
Acquisition Date: 24-Apr-18
Gate: No Gate
Total Events: 20000

Region	% Gated	% Total
R1	24.24	24.24
R2	3.65	3.65
R3	10.08	10.08
R4	62.57	62.57

e



f

Figure 2. Apoptosis of EE, NFH and doxorubicin on MCF-7 cell lines (a) control cell (b) EE ½ IC_{50} (47.22 µg/mL); (c) EE $^1/_{10}$ IC_{50} (9.44 µg/mL) (d) NHF ½ IC_{50} (48.80 µg/mL (e) NHF $^1/_{10}$ IC_{50} (9.76 µg/mL) (f) Doxorubicin ½ IC_{50} (2.9 µg/mL).

Conclusion

Based on the result, EE and NHF are potential to be developed as chemotherapeutic agents. The isolation active compounds of NHF need to be explored in detail.

Acknowledgments

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