ETHANOL EXTRACT AND ACTIVE FRACTION EFFECT OF Moringa Oleifera. Lam IN HINIBITING COX-2 ACTIVITY ON MCF-7 CELL

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ABSTRACT

The expression of COX-2 is involved in carcinogenesis through the proliferative process, angiogenesis and inhibition of apoptosis. Moringa oleifera has been shown to function as an anti-oxidant, anti-inflammatory, anti-microbial and anticancer. Moringa leaf extraction was done by percolation method, and continued to liquid-liquid extraction to obtain nonpolar, semipolar, and polar fractions using n-hexane and ethylacetate solvent. The aim of this study was to determine the effect of ethanol extract and the fraction of n-Hexane of Moringa oleifera leaf in decreased MCF-7 breast cancer cell and COX-2 expression. This study used true experimentation in vitro design using MCF-7 cells. MTT Assay was performed using a therapeutic dose 6,125-1000 μg / ml to determine IC50 in MCF-7 cells. IC50 of ethanol extract was obtained 94.44 μg / ml and 97.60 μg / ml for n-Hexane fraction. Selectivity index of both samples was 3.95 μg / ml and 2.87 μg / ml. The expression of COX-2 was tested qualitatively with immunocytochemical methods and showed that ethanol extract and n-hexane fraction of Moringa oleifera leaf were able to suppress COX-2 enzyme expression on MCF-7 cells depending on concentration.

Keywords: Moringa leaf, cancer cells MCF-7, Apoptosis and COX-2

INTRODUCTION

Cancer has a major impact on society in across the world, both men and women. 5 types of cancer which cause of death in women are breast cancer, cervix, colon, lung, and stomach 1. Based on National Cancer Institute data, side effects that may occur due to anthracycline-based chetanol extract therapy (doxorubicin) are nausea, vomiting, diarrhea, stomatitis, alopecia, susceptible to infection, thrombocytopenia, neuropathy, and myalgia 2. Several research proved that the potential of Moringa leaf as an anti-cancer agent, the content of benzyl isothiocyanate (BITC) in Moringa leaf in vitro is able to induce apoptosis against ovarian cancer cells 3. The Moringa leaf extract can decrease the activity of cancer cells HepG2 and Caco-2 4. The flavonoid content found in Moringa leaf has the potential to be an anti-cancer agent by inhibiting proliferation and inducing apoptosis of cancer cells 5. Effect Moringa oleifera leaf in inhibiting the growth of cancer cells MCF-7 has been proven, but effect of the active fraction of ethanol extract of Moringa leaf on apoptosis and expression of COX-2 on MCF-7 cells have never been done. Because of that, it is necessary to conduct research on the cytotoxic effects of ethanol extracts and its fractions on MCF-7 cells and its effects on apoptosis and COX-2 expression.
MATERIAL AND METHODS

Plant Material
The Moringa leaf sample was collected from East Dumai, Riau, Indonesia.

Extraction of Moringa leaf
An amount of 400 g dried material plant samples were crushed in a blender, then macerated in ethanol 96 % for 3 hours thereafter moved to perlocator tube. Percelation was stopped if the last 500 mg of solvent were evaporated, leaving no residuals. The solvent was evaporated at low pressure with a temperature of not more than 40°C using a Rotary evaporator. It is then taken 20 g for liquid-liquid extraction to obtain a nonpolar, semipolar, and polar fraction using n-hexane and ethylacetate solvents

Phytochemical screening Moringa leaf
Phytochemical screening carried out on moringa leaf includes examining the chemical secondary metabolites of alkaloids, flavonoids, glycosides, tannins, saponins, triterpenoids, and steroids

Exposure of Moringa Leaf Extract on Cell Culture
The ethanol extract and the fraction of moringa leaf were diluted based on selected concentration to MCF-7 cell medium. The extract of moringa leaf extract was added to the plate and allowed to stand for 24 hours. Re-washed the extract of moringa leaf extract with the cell medium.

Exposure of Moringa Leaf Extract on Cell Culture
The ethanol extract and the fraction of the moringa leaf were diluted according to the desired concentration in the MCF-7 cell medium. The moringa leaf extract was added to the plate and allowed to stand for 24 hours. Re-washed the moringa leaf extract with the cell medium.

Cytotoxic Test with Assay-MTT Method

Cytotoxic assay-MTT Method
MCF-7 cells (5x10^3 cells/well) were transferred in 96-well plate and incubated for 24 hours (70-80% confluent). Cells were treated by ethanol extract and fraction of moringa leaf, then incubated for 24 hours. At the end of the treatment incubation, MTT [3-(4,5 - dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide] 0.5 mg/ml was added to each well followed by 4 hours incubation in 37°C chamber. Viable cells react with MTT to form purple formazan crystals. After 4 hours, stopper sodium dodesil sulphate (SDS) 10% in 0.1 N HCl solution was added to dissolve the formazan crystals. Following overnight incubation (with protection from light exposure), the cells were shaken for 10 minutes before being read by an ELISA reader at λ 595 NM.

Apoptosis Test

Apoptosis Assay (Double Staining Method)
Cells (5x10^4cells/well) were seeded on coverslips (Nunc) in a 24-well plate (Iwaki) and incubated for 24 hours (50-60% confluent). Cells were then treated by ethanol extract and fraction of moringa leaf, followed by incubation for 15 hours. At the end of the incubation, coverslips containing cells were moved to object glass and a mixture solution of etidium bromide- acridine orange (Sigma, Sigma-Aldrich Corp, St. Louis, MO, USA) were added to the cells to form fluorescent cells. The fluorescent cells were examined by fluorescence microscope (Zeiss MC80) immediately. Green fluorescent cells showed viable cells, while red fluorescent cells showed dead cells.

Immunocytochemical Test
The cell suspension at each different well (density of cells 5 x 10^3 cells/well) was added 1000 μL samples in the DMEM (Dulbecco’s Modified Eagle Media) culture medium until the series reaches the end in the wells of 1/2 IC50 and 1/10 IC50. In the control group added 1000 μL culture medium. The plates were incubated in a 5% CO2 incubator for 24 hours at 37°C. The incubated cells were then harvested and crushed on the object glass to be immunocytochemically tested using a COX-2 monoclonal primer (IgG) antibody, and visualized by color reaction. The expression of COX-2 is observed using a light microscope. The cells expressing the COX-2 protein will give a brown or dark color, and the cells which did not expressing the COX-2 showed color of violet or blue. The number of cells is calculated on a certain area, both brown / dark and colored blue, then analyzed.

Data Analysis
The obtained absorbance of each well converted to percentage of viable cells :

\[
\% \text{ viable cells} = \frac{\text{Treated cells abs-Medium control abs}}{\text{Cells control abs-Medium control abs}} \times 100\%
\]

Differences in cell viability at each incubation time due to sample treatment with various series of concentrations in cell control were analyzed using SPSS 16.0 with ANOVA (Analysis of variance) and 95% Confidence Interval.

RESULTS

Extraction and Phytochemical Screening
Moringa leaf extraction results obtained extract of 74.44 g. Result of liquid liquid extraction obtained n-hexane fraction as much as 19.16 g; ethylacetate fraction 17.37 g; and water fraction as much as 15.83 g. The results of phytochemical tests obtained include ethanol extract and fraction of moringa leaf containing of flavonoids, triterpenoids / steroids and glycosides (negative water fractions). The alkaloids are positive for ethanol extracts and water fractions.

Dose of IC50 with MTT Assay
Cytotoxicity test using MTT assay methods. The concentration series of ethanol extract and fraction of cytotoxic test against MCF-7 cells was 1000; 500; 250; 125; 62.50; 31.25; and 15,625 μg / ml. The concentration of doxorubicin was 24; 12; 6; 3; 1.5; 0.75and 0.375 μg / ml. The graph of IC50 value comparison between the test materials can be seen in Figure 1.
To determine the safety of use of Moringa leaves against normal cells, cytotoxic test was also performed on vero cells which are normal renal cells of African green primates. The graph of IC<sub>50</sub> value comparison between the test materials can be seen in Figure 2. The value of IC<sub>50</sub> of ethanol extract and fraction to vero cell and to MCF-7 cells were used to calculate the selectivity index. The results of selectivity index calculation can be seen in Table 1 below.

### Table 1. Selectivity index of samples on MCF-7 cells

<table>
<thead>
<tr>
<th>S.no.</th>
<th>Extract/fraction of Moringa leaf</th>
<th>Selectivity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Ethanol extract</td>
<td>3.95</td>
</tr>
<tr>
<td>2.</td>
<td>n-hexane fraction</td>
<td>2.87</td>
</tr>
<tr>
<td>3.</td>
<td>Ethyl acetate fraction</td>
<td>2.73</td>
</tr>
<tr>
<td>4.</td>
<td>Water fraction (residual)</td>
<td>0.34</td>
</tr>
</tbody>
</table>

The apoptotic test was performed on MCF-7 cells with ethanol extract and n-hexane fraction moringa leaf as the test material because the two materials were the best tested materials with IC<sub>50</sub> among other test materials. The result of apoptotic test showed that ethanol extract and n-hexane fraction of Moringa leaf against MCF-7 cells caused late necrotic cells. The final necrotic cell present also occurs by administration of doxorubicin.

**Immunocytochemistry**

Based on observations using a light microscope with 1000x magnification, it can be seen the increase and decrease of COX-2 expression on MCF-7 cells in the control of cells without treatment and with dosage ½ IC<sub>50</sub> and 1/10 IC<sub>50</sub>, results showed in Figure 3.
The extract is said to be carbamate in the form of benzyl isothiocyanate (BITC), phenytoil isothiocyanate (PITC), and phenyl isothiocyanate (PITC). The results of COX-2 expression in the previous studies show biological activity such as antioxidant and anti-inflammatory, and anti-tumor. Isotiocyanate in Moringa oleifera (L) leaf is particularly useful as a chetanbol extractpreventive agent in cancer cells. It is in the nature in the form of benzyl isothiocyanate (BITC), phenytoil isothiocyanate (PITC), and phenyl isothiocyanate (PITC). The cytotoxicity test of extract and fraction of Moringa leaf was done on vero cell which is normal renal cell of African green ape to know the safety of Moringa leaf against normal cell. The value of IC50 extract and Moringa leaf fraction of MCF-7 cells were used to calculate the selectivity index. The results of ethanol extract selectivity index calculations are more selective than n-hexane fraction. The extract is said to be selectively high if the value of SI > 3.

Apoptosis observation was done by flowcytometry method. This method is a method to calculate five cells, necrotic cells and apoptosis quickly. In this test used a protein Annexin V that can bind specifically to phosphatidilserin contained in the cell plasma membrane during apoptosis process. DNA in damaged cells, both necrosis and apoptosis will be colored by propidium iodide (PI) which produces orange to red fluorescence. As it passes through the laser beam, the cell will excite and dissipate its light to produce fluorescence light. The apoptosis test of ethanol extract and n-hexane fraction showed cell activity to undergo late necrosis.

In the immunocytochemical staining there were five treatment groups, ie ½ IC50, ⅚ IC50, control group (medium with primary antibody treatment), and blank (medium without primary antibody treatment) and doxorubicin. The results of COX-2 expression in the
treatment group were then compared with the expression in the control group so it can be seen the effect of ethanol extract of Moringa leaves on COX-2 expression. Immunocytochemical painting results were observed using a light microscope. Positive COX-2 expression when cell cytoplasm is brown, and negative when cytoplasm is blue / dark (Fig. 3).

In the treatment group it was seen that COX-2 expression decreased with increasing levels of ethanol extract and n-hexane fraction. Decreasing the concentration of the test extract provides an increase in COX-2 expression which is inversely proportional to suppression of COX-2 expression. The flavonoid content of ethanol extract and n-hexane fraction is thought to be a decrease in COX-2 expression. The biological effects of flavonoids appear to be due to interactions with protein tyrosine kinases and cyclooxygenase enzymes. BITC reportedly suppresses COX-2 expression through inhibition of MAPKs and NF-KB signaling pathways. The resulting activity of COX is prostaglandin, which is the result of arachidonic acid metabolism. Prostaglandins play a great role in human body systems, but when overexpressed, are reported to be involved in tumorigenesis. Prostaglandins of COX-2 activity reportedly play a role in VEGF upregulation (vascular endothelial growth factor) and induce angiogenesis in tumors. VEGF alone can increase the protein and activity of COX-2 in Human Umbilical Vein Endothelial Cells (HUVEC).

CONCLUSION

From the research using ethanol extract and n-hexane fraction can be drawn the conclusion that ethanol extract and n-hexane fraction have active cytotoxic ability as anticancer compounds since they have IC50 value of 94.44 and 97.60 μg / mL. Ethanol extract and n-hexane fraction cause cells to experience late necrosis in apoptosis testing using the flowisotometry method. Ethanol extract and n-hexane fraction capable of suppressing the effect of cyclooxygenase-2 enzyme expression.


REFERENCES


