Induction of Apoptosis and Phase-Cell Cycle Inhibition of G0-G1, S, G2-M of T47D Breast Cancer Cells on Treatment with Ethyl Acetate Fraction of Jackfruit Parasite Leaves (Macrosolen cochinensis)

Roihatul Mutiah¹, Christyaji Indradmojo², Hardiyanti Held Dwi³, Tias Pramesti Griana², Anik Listyana², Ria Ramdhani¹

¹Department of Pharmacy, Maulana Malik Ibrahim State Islamic University of Malang, Jawa Timur, Indonesia.
²Department of Medical Science, Maulana Malik Ibrahim State Islamic University of Malang, Jawa Timur, Indonesia.
³Department of Chemistry, Faculty of Science and Technology, Airlangga University, Jawa Timur, Indonesia.

ARTICLE INFO

Article history:
Received on: 13/08/2017
Accepted on: 30/09/2017
Available online: 30/10/2017

Key words:
Jackfruit mistletoe leaf (Macrosolen cochinensis), T47D breast cancer, apoptosis, cell cycle.

ABSTRACT

Macrosolen cochinensis is a parasite grows in Indonesia and is known to possess the anticancer activity. This study aims to explain the anticancer activity of the extracts and fractions of jackfruit mistletoe leaves and describe the cell cycle inhibition and induction of apoptosis on T47D breast cancer cell line. The jackfruit mistletoe is extracted using the maceration method. The extract is then separated by liquid-liquid partition method with n-hexan, chloroform, ethyl acetate, and water solvent. MTT method is used to determine the anticancer activity of crude extract and fractions. Cell cycle inhibition test is conducted using flow cytometry with PI marker. The induction of apoptosis is determined by flow cytometry method using PI-Annexin V and PI double staining acridine orange-ethidium bromide. The results indicated that the IC₅₀ value of the ethanol extract, chloroform fractions and ethyl acetate fractions of jackfruit mistletoe leaves showed a higher anticancer activity with IC₅₀ which respectively are 362.8, 356.8, 314.8μg/ml. The treatment of n-hexan and water fraction show no signs of anticancer activity because it has a great value of IC₅₀ which are respectively 926.0 and 2243 μg/ml. Cell death caused by treatment of ethyl acetate fraction of jackfruit mistletoe leaf (Macrosolen cochinensis) is due to the induction of cell apoptosis and cell cycle inhibition in G0-G1, S and G2-M phase.

INTRODUCTION

Breast cancer is a malignant tumor or a collection of cancer cells developed from breast cells. It is commonly occurred in the milk ducts or lobe. WHO report that breast cancer is the leading cause of women’s death in various parts of the world. The Global cancer project (GLOBACON 2012) noted that 1,671,149 new cases of breast cancer were identified and 521,907 cases of deaths due to breast cancer occurred in the world in 2012. Breast cancer incidence in developed countries is higher, while relative mortality is greatest in less developed countries (Ghoncheh et al., 2016.)

The methods for breast cancer treatment that are widely used today are chemotherapy, radiation, and surgery. These methods aim to remove cancer tissue or kill cancer cells. However, these methods have not been optimized. They even cause side effects on normal cell’s death, which are located around the cancer cells or other organs (Lockhsin et al., 2007). Therefore, safer cancer treatment methods need to be developed. One of the cancer treatment methods that keeps being developed is the use of anticancer agents from natural materials. The use of natural materials is relatively safer because the side effects are relatively small. People may utilize something that is previously not useful to be something useful; one of them is jackfruit mistletoe. Jackfruit mistletoe (Macrosolen cochinensis) is categorized into Loranthaceae family, which usually lives on the jackfruit and wuluh starfruit host. Jackfruit mistletoe has many benefits for curing diseases and is commonly used in curing diarrhea, ulcers and headaches.

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The research conducted by Artanti (2003) indicates a result that in vitro anticancer test shows that extracts of jackfruit mistletoe has anticancer activity against T47D breast cancer cells with IC$_{50}$ = 57 µg/ml. The water extract of Jackfruit mistletoe leaf given to the mice, which is induced by benzo(a)pyrene with the dose of 750 mg/Kg BB, is effectively able to inhibit (P < 0.05) lung tumor growth with 75.27% inhibition percentage in male mice and 84.57% in female mice (Artanti, 2006).

It is also reported that at a dose of 37.5 mg/gr BB, the ethanol extract of jackfruit mistletoe leaves (Macrosolein cochinensis) can reduce the total number of lymphocytes of white rats (Rattus norvegicus) that are infected with dengue virus (Ningrum, 2010).

In this study, extraction and fractionation of jackfruit mistletoe treatment (Macrosolein cochinensis) was done to obtain fractions that have anticancer activity. These fractions were screened through apoptosis induction tests, and T47D breast cancer cell cycle inhibition test.

**METHODOLOGY**

**Research Tools and Materials**

The tools used include a set of glassware, analytical balance, clamp the wood, paper filter, clamps and static, buchner filter, rotary evaporator, vial bottle, wood clamp, vortex, 200, 1000 µl micropipette, small test tubes, small tube rack, 96-well plate, conical tube, yellow tip, blue tip, culture dish, hemocytometer, ELISA reader, a tank of liquid nitrogen, CO$_2$ - Jacketed Incubator, 96 well plate, 6 well plate, 24-well plate enclosed by coverslip, FACS Calibur, Laminar Air flow cabinet (Nuaire), vortex, centrifuge, inverted microscope, Elisa reader, fluorescence microscopy and a set of flow cytometry tools.

The materials used are jackfruits mistletoe leaves, 96% ethanol-n-hexane, chloroform, ethyl acetate, aquadest, PBS, trypsin-EDTA 1x (trypsin 0.25%), culture medium of RPMI, DMSO, 5 mg/mL MTT (50 mg MTT and 10 mL of PBS), 10% SDS in 0.1 N HCl, acridine orange, annexin V, propidium iodide (PI), and flow cytometry reagents.

**Test materials**

The jackfruit mistletoe leaves are obtained from Malang area of East Java. The plant determination is conducted at the Indonesian Institute of Sciences (LIPI) in Purwodadi, East Java. The determination specimens number 201605 is stored in Pharmacognosy Laboratory of Pharmacy Department, the Faculty of Medicine and Health Sciences (FKIK) of UIN Maulana Malik Ibrahim Malang.

**Sample Preparation**

The sample of Jackfruit mistletoe leaves is washed cleanly, then it is dried. Next, the sample is cut into small pieces and is mashed with a blender until it is smooth. It is then sieved with a size of 60 mesh.

**Maceration and Fractionation Extraction**

The jackfruit mistletoe leaves powder is added into Erlenmeyer, then it is soaked with 96% ethanol solvent and it is repeated until the color of the filtrate becomes pale. The filtrate obtained is then mixed into one and is further evaporated using a rotary vacuum evaporator to obtain a concentrated ethanol extract of jackfruit mistletoe leaves. The fractionation of ethanol extract of jackfruit mistletoe leaves is done by using liquid-liquid partition method. The concentrated extract of jackfruit mistletoe leaves ethanol is dissolved in water: ethanol (8: 2). Afterward, it is filtered, is fractionated with n -hexane in a separating funnel with a ratio of 1: 1, and is shaken sufficiently. It is put away until it forms two layers, n -hexane and water layers. This treatment is carried out several times until n-hexane layer of looks clear in order to obtain n-hexane fraction. The same treatment is applied to the water layer with several repeated fractionation using chloroform and ethyl acetate solvent with the ratio of 1: 1. The fraction result of n-hexane, chloroform, ethyl acetate, and water is evaporated with a rotary evaporator to obtain viscous fractions.

**Anticancer Activity Test**

The extract and fraction of jackfruit mistletoe leaves are made with various concentration of test solution. Doxorubicin is used as a positive control. Each concentration series was replicated 3 times. T47D cancer cell suspension in RPMI media is placed into the plate containing 96 wells and it is incubated for 24 hours. After 24 hours, the medium is discarded and is washed with PBS. The concentration series of the jackfruit mistletoe leaves extracts and fractions that is placed into the well has concentration series of 1000; 500; 250; 125; 62.5; 31.25 ppm, it is repeated 3 times (triplo), then it is incubated for 24 hours. At the end of incubation, the solution on the plate is discarded and is washed with PBS once before MTT 100 µl reagent is added into each well. The cells that have been given MTT are incubated for 2-4 hours in the incubator (until formazan salt is formed). After formazan salt is created, 10% SDS of stopper is added into 0.1 N HCl, then it is incubated in a dark place overnight. Once it is done, reading using Elisa reader with a wavelength of 595 is performed. Living cells react with MTT, forming a purple color (Mutiah, 2014).

**Absorbance and data analysis readings**

The discoloration indicator after the administration of MTT reagent leads to the absorbance readings using ELISA reader, and the percentage of living cells is calculated using the following equation:

\[
\text{Cell Viability} = \frac{(A-B)}{(C-B)} \times 100 \%
\]

Notes:
A = treatment absorbance (cell + culture medium + sample)
B = of media control absorbance (culture medium)
C = negative control absorbance (cell + culture medium)

The percentage of living cells is counted to get the number of IC$_{50}$, a concentrate which inhibits 50% growth of cell.
population, in order to find out the cytotoxic potential. The IC$_{50}$ is determined using Probit analysis (Statistic Product and Service Solution (SPSS) 16.0 for Windows).

**Double Staining Test Using Orange Acridine**

A confluent T47D cell culture is harvested and distributed using $5 \times 10^5$ cell/well concentration in 1000 μl RPMI media into plate 24 well with coverslip. After that, the cell is incubated for 24 jam hours in CO$_2$ incubator to make it adapted and normal. The next step is an extraction and active fraction to T47D cell. Then, it is incubated again for 24 hours in the CO$_2$ incubator. In the end of the process, RPMI culture media is carefully taken and washed using PBS, then the coverslip with the cell is moved onto glass object and given a reacor of orange acridine for 10 μl. The cell morphological observation employs a fluorescence microscope (CCRC, 2014).

**Cell Cycle Inhibition Test Using Flow Cytometry Method**

A confluent T47D cell culture is harvested and distributed using $5 \times 10^5$ cell/well concentration in 2000 μl RPMI media into plate 26 well. After that, it is incubated for 24 hours in the CO$_2$ incubator. Then, the RPMI media is taken by sucking it carefully and the cell is washed using PBS. The next step is the treatment of sample extraction and active fraction to T47D cell, and after that it is put into CO$_2$ incubator for 24 hours. Then, the next is the sample preparation for flow cytometry. First is the tool preparation. The media in the well is taken and put in each conical tube (1 conical per well). Then it is washed using 1 mL PBS and put in each conical tube. 250 μL Trypsin is added, then it is incubated for 3 minutes. Make sure the cell is separated one by one. 1 mL MK is added and the cell is resuspended. The cell is taken and put into conical tube by adding 2 mL PBS. Then, it is centrifuged with the speed of 2000 rpm for 5 minutes. The supernatant is taken, for apoptosis treatment 1 mL PBS is added and the cell is resuspended and moved into eppendorf. For cell cycle treatment, 1 mL cold ethanol is added and the cell is resuspended and moved into eppendorf. Each eppendorf is incubated for 30 minutes in the refrigerator. For apoptosis treatment, each eppendorf is centrifuged with speed of 2000 rpm for 3 minutes and the cell is ready to test using flow cytometer. The media in the eppendorf is moved, then 100 μL Annexin V-PI reagent and 350 μL buffer are added into the eppendorf. The cell is put into vortex to mix them well. After that, it is incubated in room temperature and dark place for 10 minutes. The cell suspension is moved into flowcyto-tube and it is ready to be injected into flow cytometry. For cell cycle treatment, 400 μL flow cytometry reagent of each eppendorf is resuspended homogeneously. Each eppendorf is wrapped with aluminium foil and given mark on its upper part. All eppendorfs is incubated in 37°C water bath for 10 minutes to activate RNase. It is resuspended before being transferred into flowcyto-tube. The cell suspension is transferred through the filter (nylon/glass fabric) using 1 ml micropipette. The lid of flowcyto-tube is holed. Flow cytometry FACS Calibur is ready to find out the profile of cell cycle. The data of flow cytometry is analysed using cell quest program to observe cell distribution during cell cycle phases of sub G1, S, G2/M and cell experiencing polyploidy (CCRC, 2014). Flow cytometry is used with 488 nm light beam and medium speed (500 cell/second) (CCRC, 2014).

**Cell apoptosis Induction Test Using Flow Cytometry Method**

Cells with $5 \times 10^5$ cell/well are planted in 6-well plate, then they are incubated until they are normal. Then, the next treatment is DMSO solvent (025%) and active isolate. After that, the cells are reincubated for 24 hours. In the end of incubation, the media is taken and transferred into centrifuge tube. Then, it is centrifuged for 3 minutes and with 2000 rpm and the supernatant is taken. For wells which media is already taken, PBS is added and transferred into the same microtube from one treatment, then it is centrifuged and the supernatant is taken. The step is repeated and the cell is harvested using trypsin. The cell transferred into the same microtube is centrifuged for 3 minutes and with 2000 rpm. The rest of harvested cells in the well is washed using PBS and centrifuged. After that, the PBS is taken. The reagent PI-Annexin V is added to the deposit carefully, and homogenized. Microtube consisting cell suspension is wrapped using aluminum foil and incubated in the 37°C water bath for 5 minutes. The cell suspension is homogenized again and transferred into flowcytometer tube using nylon filter, and it is ready to be analyzed using flowcytometer.

**RESULT AND DISCUSSION**

**Anticancer Activity Test (CCRC, 2014)**

The ethanol extract and the four fractions of jackfruit mistletoe leaves from anticancer activity test on T47D breast cancer cell line T47D using MTT method. The analysis result of cell viability shows that the increasing of the test concentration leads to the decreasing of cell viability on the treatment of mistletoe crude extract (ME), n-hexane fraction (F1), chloroform fraction (F2), ethyl acetate fraction (F3). Meanwhile, the water fraction (F4) shows that the increasing of test concentration has no influence on cell viability. The result of cell viability is presented by Figure 1.

![Fig. 1: Viability of T47D cancer cells after treatment ethanol extract 96% (EB), n-hexane fraction (F1), Chloroform fraction (F2), Ethyl Acetate fraction (F3), Water fraction (F4). Treatment was performed with 3 x replication.](image-url)
The test result of anticancer activity on breast cancer cell line T47D is analysed using Probit analysis of SPSS from cell viability data to get the value of IC₅₀. The data of IC₅₀ is presented in Table 1.

### Table 1: The value of test solution IC₅₀ on breast cancer cell T47D using Probit analysis.

<table>
<thead>
<tr>
<th>Samples</th>
<th>IC₅₀ ± SD*(μg/ml)</th>
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<tbody>
<tr>
<td>Ethanol extract 96 %</td>
<td>362.8 ± 0.78</td>
</tr>
<tr>
<td>n-Hexane fraction</td>
<td>926.0 ± 1.34</td>
</tr>
<tr>
<td>Chloroform fraction</td>
<td>356.8 ± 2.56</td>
</tr>
<tr>
<td>Ethyl Acetate fraction</td>
<td>314.8± 0.06</td>
</tr>
<tr>
<td>Water fraction</td>
<td>2243 ± 5.87</td>
</tr>
</tbody>
</table>

*The mean of IC₅₀± deviation standard, n=3.

The value of IC₅₀ reflects cytotoxicity (anticancer activity) on breast cancer cell T47D. The result of the study shows that the ethanol extract, chloroform fraction, and ethyl acetate fraction have an anticancer activity since IC₅₀ <500μg/ml. Meanwhile, the fraction of n-hexane and ethyl acetate have no anticancer activity since IC₅₀ >500μg/ml. Based on the result, the fraction of ethyl acetate of jackfruit mistletoe leaves has a stronger anticancer activity than other test solution. Therefore, the researchers conducts further study on jackfruit mistletoe leaves, namely apoptosis induction test and cell cycle regulation test.

### Double Staining Test Using Orange Acridine

The death of the cell as an impact of jackfruit mistletoe leave (*Macrosoles cochinensis*) ethyl fraction treatment through apoptosis induction on cell T47D is emphasized by the study using cell T47D with Orange Acridine-Ethidium Bromide (AO/EB coloring). The use of AO/EB aims to picture the cell with chromatin organisation changes. The use of Orange Acridine (AO) aims to show the number of the cells experiencing apoptosis. However, AO has no ability to differentiate between the living and dead cells. To get a clear picture whether the cell experiencing apoptosis but stay alive or die, the researchers use ethidium bromide which is able to intercalate with DNA and RNA and to give red color (Nafisi *et al.*, 2006; Byzkowska, 2013). The result of double staining test on ethyl acetate fraction of jackfruit mistletoe leaves is shown in the Figure 2.

![Fig. 2: The result of double staining test on ethyl acetate fraction of jackfruit mistletoe leaves using orange acridine. A) Cell control; B) The treatment using 314.8 μg/ml ethyl acetate fraction. Living cell (→a); early apoptosis (→b); late apoptosis (→c).](image)

According to the result of coloring fluorescence, it can be seen qualitatively that there is different colour between control and experimental variables which indicates the apoptosis process. The treatment of ethyl acetate fraction of jackfruit mistletoe leaf causes cell apoptosis process which is indicated by the change of shape, chromatin condense and DNA degradation that is painted with acridine orange/ethidium bromide will show bright green colour in the T47D cancer cell which experiences early apoptosis and orange colour in the cell which experiences late apoptosis process. Acridine orange is the cellular dyes that has role in living cellular dyeing (Liu *et al.*, 2015). There is an early apoptosis, late apoptosis, and apoptosis in the treatment of ethyl acetate fraction of jackfruit mistletoe leaf. Early apoptosis condition is detected by orange colour with whole orange cellular morphology. Apoptosis condition is detected by orange colour with its cellular morphology of pale orange colour. The morphological change in cell that experiences apoptosis is indicated by condensation and fragmentation of chromatin, DNA fragmentation, cellular volume shrinkage, carioptosis, and the formation of apoptotic bodies (Elmore, 2007).

### The Analysis of Cellular Apoptosis using Flow Cytometry Method

Apoptosis test using flow cytometry method aims to know the cause of cell death whether it is apoptosis or necrosis quantitatively as a treatment result of ethyl acetate fraction of jackfruit mistletoe leaf. Flow cytometry method can distinguish living cell, early apoptosis, late apoptosis and necrosis, because Reagan Annexin V and PI work selectively in bounding whole and non-intact cells (fragmentation). The result of MTT test shows that ethyl acetate fraction of jackfruit mistletoe leaf can obstruct T47D cell growth in IC₅₀ 314.8 μg/mL. Therefore, it needs flow cytometry test to know the cause of death from that treatment. The result of apoptosis test with flow cytometry is presented in Picture 1.

![Fig. 3: The result of flow cytometry test: a) T47D cell control without treatment; b) treatment of ethyl acetate of jackfruit mistletoe leaf (*Macrosoles cochinensis*) for T47D cell; c) The graphic of cell number comparison which experiences apoptosis for the cell control for 4.02% and jackfruit mistletoe 88.39%.](image)
The result of apoptosis observation (picture 3) using flow cytometry shows that treatment for ethyl acetate fraction of jackfruit mistletoe leaf in the IC$^{50}$ concentration of 314.8 μg/ml with 24 hours incubation causes 52.41% T47D cells experience early apoptosis, 35.98% of T47D cells experience late apoptosis, and 4.90% T47D cells experience necrosis. In the T47D control cell without treatment, sample experience 3.53% early apoptosis, 0.49% T47D cell experience late apoptosis, 0.19% cell experience cellular necrosis. This data prove that treatment for ethyl acetate fraction of jackfruit mistletoe leaf causes death of T47D cancer cell through mechanism of apoptosis for 88.39%.

Analysis of Cell Cycle using Flow Cytometry Method

The research result using flow cytometry of T47D cell cycle detention which is induced by ethyl acetate fraction of jackfruit mistletoe leaf using IC$^{50}$ concentration shows that T47D cell cycle detention begin to happen in induced cell of ethyl acetate fraction of jackfruit mistletoe leaf of 314.8 μg/mL in the cycle of G$_0$-G$_1$, S and G2-M phases, and experience apoptosis in G$_2$-M phase. Compare to T47D cell control shown in Picture 4a) shows that T47D cell cycle begin to happen in cycle of S and G2-M phases, and experience apoptosis in G$_0$-G$_1$ phase.

![Flow Cytometry Graphs](image)

In the Picture 4, the cell cycle above shows that treatment of ethyl acetate fraction of jackfruit mistletoe leaf for T47D cell causes change in cancer cell cycle that is indicated by the decrease of cell number in the M1 phase is increasing which means (p<0.05). The decreasing number of cell in G0-G1, S and G2M phases shows the cell cycle discontinuation. The increasing number of cell in M1 phase shows the increasing number of cells which experience apoptosis death. The mechanism of cell death caused by detention in cell cycle molecularly assumed by the role of Cyclin dependent kinase inhibitor (CDKI), is a protein that can obstruct the activities of Cdk by binding Cdk or cyclin-cdk complex. Cyclin dependent kinase inhibitor consists of two protein groups that are INK4 (p15, p16, p18, and p19) and CIP/KIP (p21, p27, p57). INK4 family forms stable complex with Cdk to prevent progression in G1 phase. CIP/KIP family regulates G1 and S phases by obstructing G1 cyclin-Cdk complex and cyclin B-Cdk1. Protein p21 also obstruct DNA synthetic by disabling proliferating cell nuclear antigen (PCNA). P21 expression is regulated by p53 because p53 is a transcription factor for p21 (Robert et al., 2013).

CONCLUSION

The result of this research is the discovery that extract ethanol, chloroform fraction, and ethyl acetate fraction show higher anti-cancer activity with IC$^{50}$ of 362.8, 356.8, 314.8μg/ml in a row. The treatment of n-hexane fraction and water fraction show null anti-cancer activity because they have high IC$^{50}$ values that are 926 and 2243 μg/ml in a row. The death of cell which is caused by the treatment of ethyl acetate fraction of jackfruit mistletoe leaf (Macrosolen cochinensis) is caused by cell apoptosis induction and cell cycle resistance in the G0-G1, S and G2-M phases.

ACKNOWLEDGEMENT

Financial support and sponsorship: We deeply thank the LPPM Maulana Malik Ibrahim State Islamic University of Malang for their financial support through “Hibah kompetitif” Research Grant in 2016 that made this study possible.

Conflict of Interests: There are no conflicts of interest.

REFERENCES


CCRC. 2014. Prosedur Tetap Uji Sitotoksik Metode MTT. Faculty of Pharmacy Gadjah Mada University, Yogyakarta. (Text in Indonesia).


Ningrum P. 2010. Pengaruh Ekstrak Etanol Daun Benalu (Macrosolen cochinensis) terhadap Penurunan Jumlah Total Limfosit Tikus Putih (Rattus norvegicus) yang Diinveksi Virus Dengue [Thesis]. Jember : Faculty of Medical University of Jember. (Text in Indonesia).


How to cite this article: