Apoptosis and Antiproliferation of Tespong Herbs (Oenanthe javanica (Blume) DC) Fraction Against MCF 7 Breast Cancer Cell

Putri Agustina [1], *Haryoto*[1], and *Peni Indrayudha*[1*]

[1]Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, Jl. A. Yani, Tromol Pos I, Pabelan, Kartasura, Sukoharjo, Jawa Tengah Indonesia 57162
*peni.indrayudha@ums.ac.id

Abstract. Breast cancer is now commonly treated with chemotherapy. The main problem in breast cancer treatment is the emergence of resistance and low efficacy of drugs. With so many side effects, it encourages many researchers to look for alternative breast cancer treatments. One possibility is the use of plant-derived natural anticancer agents, namely Herba Tespong (Oenanthe javanica (Blume) DC). Oenanthe javanica contains coumarins, flavonoids, flavonoid glycosides and polyphenols. The aim of this study was to examine the cytotoxicity, antiproliferative and apoptotic effects of a fraction active of the herb tespong (Oenanthe javanica (Blume) DC) on MCF7 cells. The antiproliferation test method used is MTT by examining doubling time values and apoptosis using double staining method. From the results of the study, the IC_{50} value of the active fraction of tespong herb was 41,35 µg/mL and was categorized as moderately active. The results obtained show that the active isolate was able inhibit the proliferation of MCF7 cells at IC_{50} concentration of 41,35 µg/ml with a doubling time of 32,32 hours. Apoptosis assays showed cell death at an IC_{50} concentration of 41,35 µg/mL.

1 Introduction

Breast cancer is the second leading cause of death in women after cervical cancer. In 2022, an estimated 290.56 million new cases will be reported and up to 43,250 people will die from breast cancer [1]. Cancer is also a leading cause of death in developing countries worldwide [2]. The main problem in breast cancer treatment is the emergence of resistance and low efficacy of drugs. Some chemotherapeutic agents, such as doxorubicin, have acquired resistance in some cases of breast cancer. Chemotherapy also causes some side effects such as the death of healthy cells around the cancer cell proliferation and causes some problems such as hair loss, nausea, vomiting and even death [3]. With so many side effects, it encourages many researchers to look for alternative breast cancer treatments. One possibility is the use of natural anticancer drugs derived from plants. Tespong herbs (Oenanthe javanica (Blume) DC) is a herbaceous plant that has been cultivated for thousands of years in tropical and temperate regions of Asia and has long been used as a traditional medicine to relieve various ailments. Phytochemical screening revealed that Oenanthe javanica contains coumarins, flavonoids, flavonoid glycosides, and polyphenols [4]. A previous study tested the phenolic acid content of Tespong herb on liver cancer cells and showed that phenolic acid had the effect of suppressing the growth of HepG2.2.15 liver cancer cells, which inhibited the growth of S-stage cancer cells. Flavonoids isolated from Tespong herb extracts had antimetastatic effects on HCT116 and HT29 colon cancers through HIF-1α inhibition mechanism, contributing to inhibition of cancer cell migration and invasion in vitro. HIF-1α (hypoxia-inducible factor-1α) is a major protein that regulates cancer cell proliferation and metastasis. HIF-1α functions as a transcription factor that plays an important role in maintaining oxygen balance [4]. In another study, when shrimp larvae (Artemia franciscana Kellogg.) were tested using the Brine Shrimp Lethality Test (BSLT) method, cell it has been shown have toxic activity [5]. This study was to determine the cytotoxicity, antiproliferative and apoptotic effects of a fraction active of the herb tespong (Oenanthe javanica (Blume) DC) on MCF7 cells.

2 Materials

The tools used in this research are rotary evaporator, water bath, FTIR, microplate 96 well, microplate 24 well, Biological Cabinet Safety (BSC), microscopyfluorescents, Pasteur pipet, incubator CO2 and, ELISA reader The materials used in this research are Tespong herb simplicia (Oenanthe javanica Blume DC), ethanol 96%, ethyl acetate, n-hexane, methanol, silica plate GF254, silica gel GF254, impregnated silica, silica gypsum, silica G60, MCF7 cells, growth media, Sodium Dodecyl Sulfate (SDS), ethidium bromide, acridine orange.

3 Methods

3.1 Extractions

Tespong herb simplicia powder was extracted using 70% ethanol at a ratio of 1:10 for three days and concentrated with a rotary evaporator until a thick extract was obtained.

3.2 Fractination

A total of 20 grams of thick extract were weighed and crushed together with 40 grams of silica gel 60 (0.063 – 0.2 mm) which had been previously activated. Grind until homogeneous. Then 175 grams of silica gel GF254 which had also been previously activated was put into the chromatography column and compacted using a vacuum. After the silica is solid, filter paper is given on top of the silica and then the mixture of extract and silica gel G60 254 is added. Filter paper is given on top of the mixture of extract and silica. Then eluted starting from the non-polar eluent first. The filtrate obtained is accommodated and numbered according to the level of polarity. The fractions obtained from KCV were then monitored by TLC. The monitoring results are then grouped based on the spots and Rf values that appear. The fraction group from monitoring I was monitored again by taking 2 vials. So fraction simplification can be done by combining fractions based on the same stain pattern and Rf from TLC results.

3.3 Cytotoxic Test

Cytotoxicity tests were performed using the MTT method associated with the CCRC. Tespong Herb fraction were dissolved in DMSO and prepared at a concentration series of 10, 20, 30, 40, and 50 µg/ml, respectively. Doxorubicin was used as a positive control and the concentration used was 25. 12.5; 6.25; 3,125 and 1,625 µg/mL. Three controls were used: cell control, media control, and solvent control. 100 µL of test solution was placed in a 96-well microplate and incubated for 24 hours in a CO2 incubator. Document the cell status of each treatment towards the end of the incubation period. Take 1 ml of MTT stock solution in PBS (5 mg/ml) diluted to 10 ml with Control Media. Incubate for 2-4 hours in a CO2 incubator to. Incubation was carried out until formazan was formed. The state of the cells was examined under an inverted microscope. Once formazan was formed, a stopper of 100 µl of SDS 10% was added in 0.1 N HCl. Plates are wrapped in paper or aluminum foil and incubated in the dark at room temperature overnight. Turn on the ELISA reader, and wait for the progressing process to finish. Inserted into the ELISA reader, the absorbance of each well was read using an ELISA reader with 595 nm. Graphs of absorbance (after deducting control media) vs concentration were made [6].

3.4 Antiproliferation Test (Doubling Time)

Antiproliferation test was done by determining the cell doubling time at the incubation time. MCF-7 cells were grown in 96 well microplates to obtain a density of 5x103 cells/well and incubated for 48 hours. The concentrations of fractions and isolates tested were IC50 values and doxorubicin with a concentration of 25; 12,5; 6,25; 3,125 and 1,625 µg/mL. After being given the treatment, the cells were incubated in a 5% CO2 incubator at 37^{o}C with observations at 24 hours, 48 hours, and 72 hours. Then the absorbance was measured using an ELISA reader at a wavelength of 595 nm. From the results obtained, a graph is made between the log number of live cells and the length of incubation time, then the time difference is determined to reach the number of 2 times the initial cells (knowing the doubling time). From the equation obtained, the doubling time value can be determined (Ismaryani et al., 2018).

3.5 Double staining

The 24 well microplate containing cells was discarded by control media with a Pasteur pipette. Wash the cells with 500 l of PBS each, remove and add a PBS with a Pasteur pipette. Samples with a concentration of IC50 were put into wells of 1000 µL/each. The control cells were filled with DMEM media and the solvent control was filled with DMSO. Plates were incubated for 10 hours, and cell conditions were observed under a fluorescent microscope. After the incubation period was completed, the media in the wells was discarded and each well was washed with PBS. The cover slip is taken using tweezers and placed on a glass object. Add 10 µL of ethidium acridine orange on top. Observations were made with a fluorescent microscope and documented [6].

3.6 Data analysis

The cytotoxic test is calculated from the results of absorbance readings with an ELISA reader converted into % of live cells.

Based on the % live cells data, the IC50 value can be calculated using a linear regression equation which is the relationship between % live cells vs. extract/fraction content of tespong herbs. The smaller the IC50 value, the more potential the extract has in inhibiting the growth of MCF 7 cells.

After obtaining the % of live cells, IC_{50} was calculated using probit on SPSS 18.0 [7]. Then an unpaired t-test was performed using the SPSS program to determine the significant difference between the results of the treatment of the sample and the positive control (Doxorubicin). The data is declared significant if the value of <0.05 [8]. For the antiproliferation test, the absorbance obtained from the ELISA reader at a wavelength of 595 nm, a graph was made between the log of the number of living cells and the length of incubation time, then the difference in time was determined to reach 2 times the number of initial cells (to know the doubling time). From the equation obtained, the doubling time value can be determined [9].

4 Results and Discussion

4.1 Extraction

Extraction is done by the maceration method. This method was chosen because the process is easy, simple, and does not go through a heating process to prevent damage to compounds that are not heat resistant [10]. The solvent used is 95% ethanol, chosen because of its polar nature so that it can attract the compounds contained in the herbal tespong simplicia [11]. A total of 700 grams of tespong herb simplicia were soaked in 95% ethanol for 24 hours. After 24 hours, the filtrate was filtered and the filtrate was collected, then the simplicia dregs were repressed again for 24 hours to obtain an almost colorless filtrate. The filtrate obtained was then concentrated with a rotary evaporator at a temperature of 60^{o}C to remove the ethanol solvent contained in the filtrate. The total viscous extract obtained was 83,5 grams with the extract yield value obtained is 11.92%. The size of this yield value is influenced by the amount of secondary metabolite

content in the studied extract [12]. After obtaining the thick extract, then the fractionation process was carried out using the Vacuum Liquid Chromatography (KCV) method.

4.2 Fractionation

From the results of the fractionation, obtained 14 fractions were which were then monitored through thin layer chromatography to classify the filtrate into simpler ones. The following are the results from monitoring the fraction seen under a 254 nm UV lamp.

The spots with similar Rf values in TLC monitoring were combined to obtain 3 main fractions, namely the non-polar fraction, the semi-polar fraction, and the polar fraction. In fractions number 1 and 2, there were no spots. The principle of KCV is partition and adsorption where the separation uses the help of pressure from a vacuum pump. The stationary phase in the KCV fractionation used Silica G60 GF254 and the mobile phase used a solvent with increasing polarity. The use of solvents with increased polarity is intended so that each solvent can dissolve compounds based on their level of polarity. From the results of the fractionation, 14 fraction reservoirs were obtained which were then monitored by TLC.

4.3 Cytotoxic Activity Test

A cytotoxic activity test was carried out using the MTT method. The principle of the MTT method is to measure the activity of mitochondrial dehydrogenase in living cells that can convert MTT into formazan salts [13]. The test samples used were the ethyl acetate fraction of tespong herbs with a concentration series of 10, 20, 30, 40, and 50 µg/mL. A cytotoxic activity test was carried out in vitro using MCF 7 breast cancer cells.

Cells were counted using a hemocytometer and observed under an inverted microscope. The results of the cell count obtained are 139 x 10^4 /mL. After being incubated for 24 hours, the cells were observed under an inverted microscope. Dead cells are round and floating, but live cells are oval needles that stick to other cells around them. The outcome of treatment cannot be determined by microscopic observation alone. Further testing, such as the addition of MTT reagent, should be performed. In this method, living cells absorb MTT reagent, forming purple formazan crystals that are insoluble in water. Violet intensity was measured by its absorbance value using ELISA reader at a wavelength of 595 nm. Data obtained in the form of absorbance and converted to % viable cells are presented in Tables 1.

Tabel 1. Viable Cell Percentage of Tespong Herb Fraction and Doxorubicin

Sample	Concentration	% Viable Cell ± SD
Active Fraction	10	61,14 ± 0,89
	20	57,46 ± 0,89
	30	53,59±1,21
	40	50,78 ± 0,73
	50	47,19±0,61
Doxorubicin	25	50,48 ± 0,89
	12,5	48,64 ± 1,18
	6,25	45,93 ± 0,50
	3,125	43,89 ± 0,58
	1,625	39,92 ± 0,93

From the results of cell viability, the IC50 value can then be calculated using a linear regression equation. The IC50 for the N-Hexane fraction was 41,35 µg/mL and the IC50 value was 2,06 g/mL for Doxorubicin.

The principle of the MTT method is to measure the activity of mitochondrial dehydrogenase in living cells, which can convert MTT to its formazan salt. From the results of cell viability, the IC50 value can be calculated by used a linear regression equation. The IC50 result for the active fraction was 41,35 µg/mL and the IC50 value was 2,06 µg/mL for Doxorubicin. According to the National Cancer Institute (NCI), anticancer activity is divided into into three categories, namely active with IC50 values of less than 20 µg/ml, moderately active categories with IC50 values of 20–100 µg/ml, and inactive categories with IC50 values >100 µg/mL. Based on the categories above, the tested tespong herb fraction were placed in the moderately active category.

Groups of fractions doxorubicin were retested with unpaired t-tests to confirm significance between the two test samples. The results obtained were p-value = 0.05, indicating there is significant difference between the IC50 values of the fractions and doxorubicin ($p>0.05$).

Based on the results of previous research, which was carried out by [5] cytotoxic test of tespong herb ethanol extract using the BSLT (Brine Shrimp Lethality Test) method on Shrimp Larva (Armenia franciscana Kellog.) obtained an IC50 value of 91,20 ppm and categorized as moderately active. Oenanthe javanica belongs to the Apiaceae family. In a previous study, Seseli petroleum M. Bieb, which was tested on MCF7 cells, showed an IC50 yield of 390, 38 g/mL[14]. Anethum graveolens was tested on MCF7 cancer cells and the IC50 value was 104 g/mL. The method used is the MTT test. In HeLa cancer cells it was 122 µg/mL and in A-549 cells it was 156 µg/mL. Anethum graveolens, which are still included in the Apiaceae family, were also tested for cytotoxicity and antiproliferation on MCF7 cancer cells and obtained an IC50 value of 67 µg/L [15]. It is known that the compounds contained in A. graveolens are essential oils with the main constituent being carvone (51%) [16].

4.4 Antiproliferation Test

The doubling time method was used to determine the antiproliferative effects of Tespong herb isolates. This procedure is performed according to the MTT procedure tested for cytotoxicity. Doubling time is the time it takes for a cell to grow to twice its original number. Samples was used, namely the N-hexane fraction from Tespong herb, four test concentrations were used, namely IC_{50}, 1/2 IC_{50}, 1/4 IC_{50}, and 1/8 IC_{50}, and incubation times varied for 24 hours, 48 hours, and 72 hours. The results of the doubling time study are shown in Table 2.

Table 2. Result of Doubling time Tespong Herb Fraction

Sample	Concentration (µg/mL)	Doubling Time (Hours)
Tespong Herb Faction	IC_{50}	32,32
	1/2 IC_{50}	31,27
	1/4 IC_{50}	30,96

	1/8 IC_{50}	30,64

Doubling time is the time required for cells to grow to twice their initial number [17]. Based on the results obtained, it can be seen that the doubling time value of the tespong herb isolate was greater than that of the Tespong herb fraction. The herbal isolate of tespong was said to be better because it had a longer time than the N-Hexane fraction, thus slowing down cell proliferation. The two test compounds also showed an increase in the doubling time value along with the concentration. This indicates that the higher the concentration, the greater the inhibitory effect of MCF7 cell activity [18]. The time required for MCF7 cells to multiply by themselves is 24 hours [9]. The addition of isolates proved to be able to inhibit cell proliferation time longer than the addition of fractions.

4.5 Apoptosis Test

The test concentration used for the apoptosis test was the previously obtained IC_{50} value of the tespong herb fraction was 41,35 µg/mL. Based on the results of the apoptosis test, it was found that green fluorescent cells indicate that the cells are alive, while the dead cells will fluoresce to orange/red.

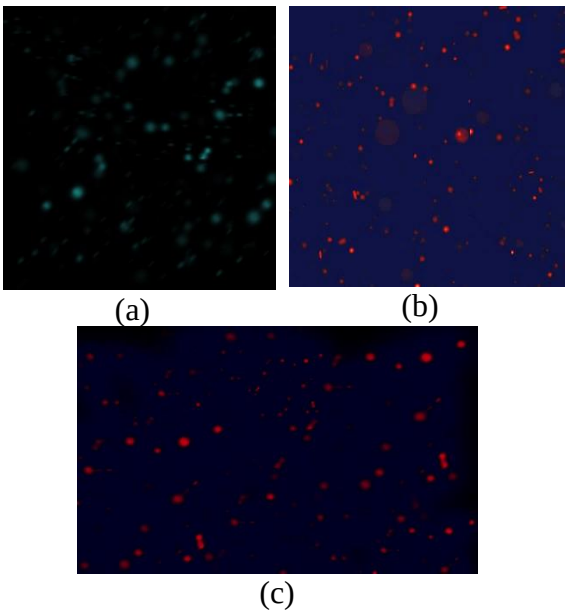

Figure 1Apoptosis of MCF 7 Cancer Cells with (a) Control Cells, (b) Tespong Herb Fraction, and (c) Doxorubicin Positive Control

Visual observations of cell apoptosis were carried out with the addition of an acridine orange - ethidium bromide reagent [19]. When observed under a fluorescence microscope, cells undergoing apoptosis will be orange in colour due to the bond between ethidium-bromide and fragmented DNA from cancer cells undergoing apoptosis, and living cells will show a green colour [20]. The orange colour formed indicates that the cell undergoes late-phase apoptosis in the cell death cycle. Cell necrosis of normal cell size is also marked in orange. Induction of apoptosis is one of the promising forms of treatment in cancer treatment. Induction of apoptosis will cause cancer cells to increase their apoptotic ability and inhibit the proliferation of these cancer cells. This treatment strategy by induced apoptosis will cause the cell apoptotic pathway to become normal so that it can inhibit the formation of cancer in the body [21]. From the results of the apoptosis test, fractions with a concentration of IC_{50} were able to induce apoptosis of MCF7 cells. This can be seen in Figure 1(b and c) where the orange fluorescent cells were treated with Fraction and doxorubicin, while the control cells were green fluorescent (Figure 1(a)).

5 Conclusion

The content of active compounds in tespong herbs (Oenanthe javanica (Blume) DC. is thought to be a group of phenolic compounds. In previous studies, it was stated that the ethanol extract of tespong herbs contained chlorogenic acid compounds seen from the results of FTIR identification showing similar results as evidenced by the wave numbers formed The cytotoxic activity using the MTT method showed promising results where the IC_{50} result of the active fraction of tespong herb obtained was 41,35 µg/mL. According to the National Cancer Institute (NCI), the isolate of the tespong herb tested was in the moderately active category. ethanol extract of tespong herb (Oenanthe Javanica) against MCF7 cells showed the ability of active isolates to prolong the doubling time of MCF7 cells for 320 hours with a concentration of IC_{50}. For the apoptosis test, it was shown that the addition of tespong herb fraction was able to provide cell death which was indicated by a fluorescence colorless which is seen under a fluorescent microscope. So from the three types of tests that have been carried out, the tespong herb has a good potency as anticancer.

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