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INTERNATIONAL JOURNAL OF PHARMACEUTICAL RESEARCH

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IJPR INCLUDED IN UGC-APPROVED LIST OF JOURNALS - REF. NO. IS SL. NO. 4812 & J. NO. 63703

Published by : Advanced Scientific Research

ISSN 0975-2366

Volume 10, Issue 3, July - Sept, 2018

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Published by : Advanced Scientific Research

ISSN 0975-2366

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ISSN 0975-2366
https://doi.org/10.31838/ijpr/2018.10.03.072

Research Article

STERCULIA QUADRIFIDA R.BR ETHYL ACETATE FRACTION INCREASES CISPLATIN CYTOTOXICITY ON T47D BREAST CANCER CELLS

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Received: 16.06.18, Revised: 24.07.18, Accepted: 02.09.18

ABSTRACT

Cisplatin is a chemotherapeutic agent used as cancer cure with side effects and resistance. Ethyl acetate fraction of faloak bark (EAFB) are proven to have cytotoxic effect to cancer cells. This research aims to review the combination effect of cisplatin and EAFB to the sensitivity increase on breast cancer cells, which will be confirmed through apoptosis induction and cell cycle modulation. The cytotoxic effect was tested using MTT assay on T47D cell by using IC_{50} parameter. The combination was tested by determining their combination index (CI) and cell viability. The combination effect in apoptosis induction and cell cycle modulation were observed using flow cytometry method. The cytotoxic test result of the combination shows CI value of below 1 at the concentration of EAFB as much as 6 µg/mL, and cisplatin as much as 2,5 µM. The combination of EAFB and cisplatin results in phase S cell accumulation (29,98%) on breast cancer cell T47D and was able to induct apoptosis. It is proven that ethyl acetate fraction of faloak bark can be developed as a co-chemotherapeutic agent with cisplatin to increase the effectivity of breast cancer treatment.

Keywords : Cisplatin, EAFB, cytoxicity, cell cycle, apoptosis.

INTRODUCTION

Cancer was the major mortality cause in the world with the number of 8.2 million deaths in 2012 and predicted to increase continuously from 14.1 million new cases in 2012 to 22.2 million in 2030 [1,2]. Breast cancer was the fifth major causes of total cancer deaths after lung cancer, liver cancer, stomach cancer, and colorectal cancer, but it was the main cause of cancer deaths among women in the world. There were estimated 555.000 women deaths due to breast cancer in 2012 [2].Nowdays, chemotherapy is a common strategy for treating breast cancer after surgery [3]. Chemotherapeutic agents usually show a low selectivity properties due to antiproliferative properties against both cancer and normal cells4. Moreover, chemotherapeutic agents exhibit some negative reaction such as narrow therapeutic index, induce multidrug resistance (MDR) *via* several molecular changes [5], and harmful side effects on cardiovascular system [6]. More selective chemotherapeutic agents development has been done by the production of trastuzumab for HER2 positive breast cancer treatment [7] and everolimus for HER2 negative breast cancer treatment [8]. However, breast cancer therapy using a conventional chemotherapeutic agent is still widely used due to economical consideration.Cisplatin is a chemotherapeutic agent used in breast cancer therapy as monotherapy or in a combination [9]. Cisplatin induces side effects such as neurotoxicity, nephrotoxicity [10], and bone marrow suppression. Besides that, usage of cisplatin as chemotherapeutic agent tends to result an incidence of drug resistance. The drug resistance associated with cisplatin is occurred through changes in: cellular uptake, drug efflux, inhibition of apoptosis, and regulation on DNA repair. Side effects and resistance due to cisplatin administration can be occurred when a high dose of cisplatin is given to reach more effective treatment [11]. Therefore, further study is needed to discover a more effective and selective breast cancer treatment method. Indonesian medicinal plants, *Sterculia quadrifida* R.Brprovide high potency to be developed as novel breast cancer chemo-preventive agents. Extracts and ethyl acetate fractions of T47D breast cancer cells showed that ethyl acetate fraction have a high cytotoxicity and selectivity effect [12]. Administration of chemotherapeutic agents in a combination provide a synergistic effect, increase sensitivity of cancer cells and further reduce dose of each chemotherapeutic agent to be used [13]. Based on the previous researches, the ethyl acetate fraction is potential to be combined with cisplatin as chemotherapeutic agent for breast cancer treatment. The combination between the ethyl acetate fraction with cisplatin is expected to be able to reduce cisplatin dose and thus will be able to alleviate the side effects and breast cancer cell resistance caused

by cisplatin administration. In this research, there was found that combination between ethyl acetate fraction with cisplatin showed cytotoxic activity to T47D cells through the cell cycle modulation and apoptosis induction.

Materials and methods

General experimental procedures

Materials used in this research are *Sterculia quadrifida* R.Brbark obtained from ende, Timor island. The concentrated ethyl acetate fraction were used as in vitro cytotoxic test samples. The concentrated extract was diluted with dimethyl sulfoxide (DMSO) with the concentration of DMSO as much as 0.1% in the culture medium. The chemotherapeutic agent used was cisplatin (Wako). T47D cell was grown in the high glucose DMEM (Dulbecco's Modified Eagle Media) culture (Gibco) which contains 10% Fetal Bovine Serum (FBS) (v/v) (FBS Qualified, Gibco, Invitrogen USA), 1.5% penicillin-streptomycin (v/v) (Gibco, Invitrogen USA) and 0.5% Fungizone (v/v) (Gibco). The cell harvesting from Tissue Culture Dish (IWAKI) used 0.25% trypsin-EDTA (Gibco, Invitrogen Canada). Cytotoxicity test using MTT assay. The reagent used was 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, USA). MTT was made with the concentration 5 mg/mL, diluted in phosphate buffer saline (PBS) pH 7.4. Working reagent was made by diluting the stock ten times using the culture medium. PBS pH 7.4 was made by diluting 8 g NaCl; 0.2 g KCl; 0.2g KH2PO4; 1.15 g Na2HPO4 in 1 L of bidistilled water. Stopper reagent contains 10% (w/v) Sodium Dodecyl Sulphate (SDS) (Merck-Schuchardt, Germany) in 0.01 N HCl (Merck, Darmstadt, Germany). Cell cycle materials used Propidium Iodide (PI) solution in PBS which contains 1 mg/ml (minimum 95% (HPLC), Sigma-Aldrich Co., St Louise, MO, 63178, USA), 10 mg/ml RNAse (obtained from Laboratory of Animal Sciences, NAIST, Japan) and 0.1% (v/v) Triton-X 100 (triton X-100 for GC, E. Merck, 64271, Darmstadt, Germany). The apoptosis material used was annexin V-FLOUS Apoptosis Detection Kit (Roche, USA).

Instruments

The instruments used in this research was autoclave (Hirayama HV 25 020585175, Hirayama Manufacturing Co., Japan), liquid nitrogen, Labconco purifier class II biosafety cabinet (Delta Series, Labconco Corporation, Missouri, USA), CO_2 incubator (Heraeus), inverted microscope (Nikon, Eclipse, TE 2000-U), hemocytometer (Nebauer improved 0.100 mm Tiefe Depth Profondeur 0.0025 mm2, Germany), cell counter, micropipette (Pipetman®neo Gilson, France), digital camera (Sony), centrifuge (Sigma 203, B.Braun Biotech International), digital scale (Mettler Toledo, AG204 Delta Rang®), stirrer (Nuova, Thermolyne), mixer (Maxi Mix II, Thermolyne type 37600 mixer, Iowa, USA), oven (Memmert), ELISA reader (Bio-Rad microplate reader Benchmark serial no. 11565, Japan), FACTScalibur flow cytometer.

Extraction and Fractination

The bark of *Sterculia quadrifida* R.Brwas cleaned and washed with running water, then dried under 50 °C for 4 days. Bark was grinded to form powder. Maceration method was used to extract using 96% ethanol as solvent. About 250g of the powder were weighed and diluted in 1 L of ethanol and macerated for one day. On the next day, extract filtering was done using flannel cloth. The extract obtained was then evaporated to separate ethanol and the extract of both plants. The precipitate was then re-macerated. After the extract thickened, extract fractionated with ethyl acetate solvent with liquid-liquid extraction method. Fractionated liquid dryed.

Identification of chemical compounds in fraction

One milligram of were weighed and diluted in 1 mL of ethyl acetate. The diluted fraction is then spotted on a plate. The mobile phase used was chloroform-ethanol 9:1 (v/v). Then, a chamber to place the mobile phase was prepared and TLC (thin layer chromatography) test was performed. The TLC plate was inserted and set aside until the mobile phase reached the top. After the mobile phase reached the top, the plate was removed from the chamber and was sprayed with cerium sulphate and Dragendorff reagents. The plate is then dried briefly in the oven and the spot formed is used to calculate the hRf value.

Cell preparation and harvesting

The cell suspension was grown in the Tissue Culture Dish (TCD) and was incubated in the CO_2 incubator with a temperature of 37°C. The cell condition was then observed under the microscope and then was incubated in the 5% CO_2 incubator. After the cell became confluent (±80%), cell harvesting was done by removing the culture, washing the cell using 3 mL PBS 2 times, and then adding 0.25% trypsin-EDTA so that the cell was able to be released from the TCD. After 30 s, the 0.25% trypsin-EDTA was removed and the cell was incubated for 1 min in the CO_2 incubator. 2-3 mL of media was added and then re-suspended so that the cells detached one by one. The cell suspension was then transferred into a new, sterile conical tube. The number of cells was calculated by using hemocytometer and cell counter and then, cell suspension was made with the needed concentration. The single and combination cytotoxicity test used the cell density of 8×10^4 cells per well plates. All the tools used for cell preservation was sterilized and sprayed with 70% alcohol when they were inserted in the LAF. Throughout the research, LAF was conditioned to be always sterile by spraying 70% alcohol.

The preparation of test solution

Test solutions stock which are EAFB was made by diluting 5 mg of the fraction in 50 μL of DMSO so the concentration of 100.000 μg/mL was obtained.

The concentration of the cisplatin solution stock was 1000 μM. The solution stock of EAFB was diluted using culture medium to the concentration of 1, 10, 25, 50, 100, 75, and 200 μg/mL to be used as the single cytotoxicity test solution. The cisplatin test solution was diluted using culture medium to the concentration of 1, 2, 5, 10, 15, 30, and 50 μM. The two combination treatment between extracts of EAFB was made in several concentrations, which are 1/12, 1/6 and 1/3 of the IC_{50} value. Apoptosis was done using flow cytometry with the EAFB and cisplatin concentration enough to obstruct the growth up to 50% of cell population. Certain concentration was used for both the single and combination treatment on the apoptosis test and cell cycle test. The observation for apoptosis test was done in the 24 h of the incubation time.

Single and combination cytotoxicity test using MTT assay

The cells were harvested with the concentration of 8×10^3 cells per well plates and was diluted with the culture medium (CM), and was planted into microplate 96 wells much as 100 μL/well plate and was incubated for 24 h in a 5% CO_2 incubator. Before being used for treatment, the media in the plate was removed and the plate was washed using PBS as much as 100 μL/well plate. Then the PBS was removed and 100 μL/well plates of test solution was added. The cells were then incubated further for 24 h. After incubation, PBS was used for washing and MTT reagent was added as much as 100 μL/well plates followed by 3-4 h of incubation at 37 °C. Afterwards, stopper reagent was added (10% SDS in 0.01 N HCl) as much as 100 μL/well plates and continued with an overnight incubation at room temperature and dark condition. ELISA reader was used to read the absorbance of life T47D cells at the wavelength of 595 nm. Single treatment absorbance data was converted into the viability percentage and used to calculate the IC_{50} value. After the IC_{50} value was known, cytotoxicity test was conducted to the combination of EAFB with the chemotherapeutic agent cisplatin in various combination ratios. Cytotoxicity test of EAFB with cisplatin was done with the concentration below IC_{50} as shown in Table 1.

Table 1 : The ratio of concentration used in the combination of EAFB with cisplatin

EAFB (1/12 IC_{50}); C (1/12 IC_{50})	EAFB (1/12 IC_{50})	C (1/12 IC_{50})
EAFB (1/6 IC_{50}); C (1/6 IC_{50})	EAFB (1/6 IC_{50});	C (1/6 IC_{50})
EAFB (1/3 IC_{50}); C (1/3 IC_{50})	EAFB EAFB (1/3 IC_{50})	C (1/3 IC_{50})
CC	CC	MC

EAFB:Ethyl acetate fraction *Sterculia quadrifida* R.Br; C: Cisplatin; CC: Cell Control; MC: Medium Control.

Cell preparation for apoptosis and cell cycle observation using flow cytometry assay

As much as 5×10^5 cells/well plates were cultivated in 6 well plates, each in 1000 μL, then incubated overnight and was observed until the cells were ready to be treated. The cells were treated with EAFB and the combination between the two and cisplatin with a chosen concentration series. For the combination treatment, 300 μL of EAFB was added with 300 μL of cisplatin with a concentration series, while for the single treatment, 900 μL of EAFB and cisplatin was added into the well plates following a concentration series. For the control cells, 900 μL of culture medium was added into the well plates. After treatments, all cells were incubated for 24 h.At the end of incubation, cell mediums were transferred into conical tubes and washed with 500 μL of PBS into every well plates, then the PBS was transferred into the same conical. 0.25% trypsin-EDTA was added as much as 200 μL/well plates for harvesting followed by incubation for 3 min so that the cells are separated from the plate base. 1000 μL/mL of culture medium was added and the cells were resuspended to become single cells to be transferred into the same conical. The cells were centrifuged with the speed of 2000 rpm for 5 min and the culture medium were removed. The cell precipitate formed were placed in conical tubes with aluminum foil as its cover and was diluted with annexin V-FLOUS buffer kitby adding 2 μL PI and 2 μL annexin V. The cell suspension was homogenized and incubated for 10 min at room temperature with the conicals covered by aluminum foil. The cells were transferred into flow cytometry tubes and analyzed.

Statistical analysis

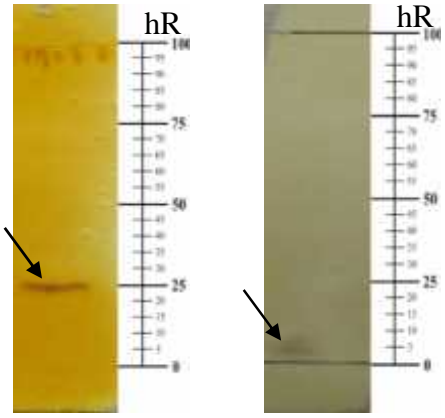
IC_{50} calculated with probit analysis method. The obtained absorbance were converted to percentage of living cells. Synergistic cytotoxic determined by calculating the combination index (CI combinatorial methods or index) by Chou [14] and drug reduction index (DRI) using software CompuSyn (www.combosyn.com) and the resulting isobologram. Flowcytometry data shows the percentage of cells contained in four quadrants, namely LL (lower left), LR (lower right), UL (upper left), and UR (upper right). LL quadrant shows the percentage of living cells, LR quadrant shows the percentage of cells undergoing early apoptosis, UL quadrant shows percent cell necrosis, UR quadrant shows percent late cells undergoing apoptosis. Induction of apoptosis known to compare the effects of single compounds and combination treatment with control cells. Data were

analyzed using flowcytometry flowing. To see the distribution of the percentage of cells in each phase G1, S and G2 / M. The inhibition of the cell cycle can be determined by comparing the treatment effect of the test solution with control cells.

Results

Identification of chemical compounds in EAFB

The identification of chemical compounds in EAFB was done to ensure that fraction contain alkaloids and terpenoids which are scientifically proven to have cytotoxic properties. The test result on EAFB (Fig 1.) shows one brown spot after being sprayed with cerium sulphate reagent that shows hRf 5 indicates that EAFB contains carbon. After Dragendorff reagent was sprayed onto the plates, a spot with hRf of 25 in reddish orange. The spot indicates positive alkaloids if the color was brownish orange after being sprayed with Dragendorff reagent. Dragendorff (BI_3KI) is a reagent widely used in identifying alkaloids where heavy metal in the Dragendorff will create a bond with a lone pair electron in the N atom of alkaloids [15].



(a) (b)

Fig. 1: Result of Dragendorff test result (a), cerium sulphate test (b) on EAFB
Elution was done with 60 F254 silica gel as stationary phase and the mixture of chloroform : methanol (9:1, v/v) as mobile phase. Detection was done in the visible light range.

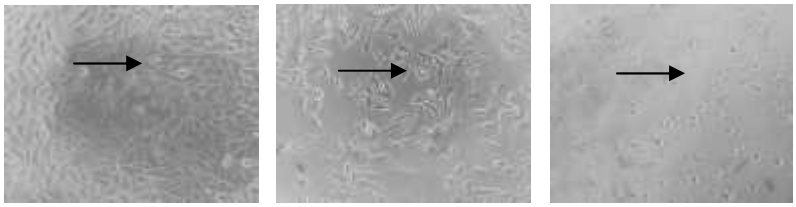
Single cytotoxicity test result on the EAFB with cisplatin at the T47D breast cancer cells

Cytotoxicity test was done to determine the potential of EAFB with cisplatin in inhibiting T47D breast cancer cell. Before the cytotoxicity test was done on the combination of the three, the individual IC_{50} value was calculated to determine the concentration suitable for each component based on their IC_{50} value. In this research, treatment was done on T47D cells in DMEM high glucose medium with a 24 h incubation. The cytotoxic effect of EAFB and cisplatin was shown with the decrease of cell viability and morphological change on T47D breast cancer cells.

Table 2: IC_{50} value of EAFB and cisplatin on T47D cells

Sample	Linear Regression Equation	IC_{50} (Mean ± SD) n = 5 experiments
EAFB	y= -45,93 x + 46,76	14,35 ± 0,54 μg/mL
Ciplatin	y= -74,74x + 87,96	15,82 ± 0,75 μM

SD: Standard deviation, IC_{50} =Inhibitionconcentration50%



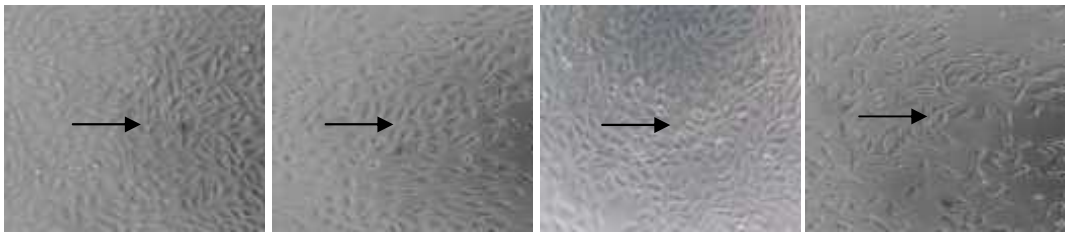
(a) (b) (c)

Fig. 3.Treatment effect on T47D cells

As much as 8000 cells per well plates in 96 well plates were incubated for 24 h in DMEM Hi-glucose medium. Observation was done under an inverted microscope with a magnification of 100 times. (a) control; (b) 15 μg/mL EAFB; (c) 10 μg/mL cisplatin. The IC_{50} value was calculated from the linear regression between log of the concentration *versus* % viability with the confidence value of 95% ($p<0,05$). The treatment with the EAFB (Fig. 3b), and cisplatin (Fig. 3c) shows a decrease the number of living cells compared to the control (Fig. 3a). The cells look round and fragmented which indicates a change in cell morphology, but it is yet to known whether the cell death was caused by a necrosis or apoptosis process, with proliferation as its inhibition process.

Cytotoxicity test result on the combination between EAFB and cisplatin on T47D breast cancer cells

The cytotoxicity test on the combination was done to analyze the effect of adding the EAFB into the combination with cisplatin on T47D breast cancer cells. The concentration ratio used for the combination were 1/12, 1/6, and 1/3 of the IC_{50} value. The concentrations were of a lower concentration compared to IC_{50}. The combination is expected to be able to reduce the clinical side effects from the use of a chemotherapeutic agent, so it was done by reducing the concentration of cisplatin as the chemotherapeutic agent. The combination index (CI) value was the parameter used to see the effect of the combination between the EAFB and cisplatin. The efficacy classifications produced were synergistic, additive, or antagonistic. The cytotoxicity test for the combination was done using MTT assay. The concentration series of the combination for the EAFB was 2.5, 5, 10 μg/mL and cisplatin was 1.25, 2.5, 5 μM. The cell morphology changes of T47D cells caused by the combination between the EAFB with cisplatin shows shrinkage and cell morphology changes (Fig.4a-d). Combination EAFB with cisplatin at concentration ratio of 1/12, 1/6, and 1/3 resulted the CI value not more than 1.00 (Table 3), so it proved that these combinations exhibited a synergistic effect.



(a) **(b)** **(c)** **(d)**

Fig. 4: The effect of treatment of the combination between the EAFB with cisplatin towards the growth of T47D cells.

As much as 8000 cells per well plates in 96 well plates were incubated for 24 h with or without treatment in DMEM Hi-glucose medium. Observation was done under an inverted microscope with a magnification of 100 times. (a) control; (b) combination ratio of 1/12 IC_{50}; (c) combination ratio of 1/6 IC_{50}; (d) combination ratio of 1/3 IC_{50}. The CI value of the combination between the EAFB with cisplatin shows synergistic effect ($CI<1$).

Table 3: The combination index (CI) value of the combination between cisplatin with EAFB on T47D cells

Concentration Ratio	Cell Viability (%) (Mean ± SD) n = 5 experiments	CI
1/12 IC_{50}	52.86 ± 0.56	0.43
1/6 IC_{50}	45.76 ± 0.88	0.58
1/3 IC_{50}	39.87 ± 0.97	0.86

SD: Standard deviation, CI =Combination Index

The modulation of cell cycle from the combination of EAFB and cisplatin on T47D breast cancer cells

The DNA synthesis on the cancer cells goes through a cell cycle, as the one on normal cells do. One of the main targets in inhibiting the proliferation of cancer cells is by the modulation of cell cycle that can be observed through the flow cytometry method. Flow cytometry is able to detect each phase in the cell recycle based on the number of chromosomes in each phase (G1, S, and G2/M). The G1 phase has 2n (diploid) chromosome, S phase undergoes replication in the preparation of going into the G2 phase so the number of chromosome sets is between 2n and 4n, while G2 phase creates 4n (2 diploid cells) chromosome. Propidium iodide was used to color each phase since it has the ability of interacting with DNA [16]. The observation of cell cycle profiles was done at the 24th h. Flow cytometry analysis using

the flowing program is shown in figure 5 and the detailed distribution percentage of cell cycle is shown in Table 4.

Table 4 : Percentage of cell cycle distribution after treatment

Sample	G1 phase (%) (Mean ± SD) n = 5 experiments	S phase (%) (Mean ± SD) n = 5 experiments	G2/M phase (%) (Mean ± SD) n = 5 experiments
Control	48.98 ± 1.22	11.62 ± 1.45	24.68 ± 0.88
EAFB (6 μg/mL)	34.83 ± 0.60	13.82 ± 0.64	24.83 ± 0.43
Cisplatin (2.5 μM)	43.87 ± 0.86	11.24 ± 0.87	29.93 ± 0.65
6 μg/mL EAFB + 2.5 μMof Cisplatin	24.82 ± 0.65	29.98 ± 0.97	15.98 ± 0.39

SD: Standard deviation

The cell control undergoes a cell distribution in G1, S, and G2/M phase. Research shows that the EAFB results in the accumulation of cells in the S and G2/M phase. Cisplatin results in the accumulation of cells in the S phase (Fig. 5). The combination of the three results in the accumulation of cells in the S phase compared to the cell control. With the combination treatment, the percentage of cell cycle distribution on the S phase was 29,48% higher than the treatment of only cisplatin with 11.24%. The cell accumulation on the S phase in the combination treatment shows an increase compared to the cells without treatment (control cells) from 11.62% to 29.98%. The cell accumulation was caused by cell cycle arrest in the said phase.

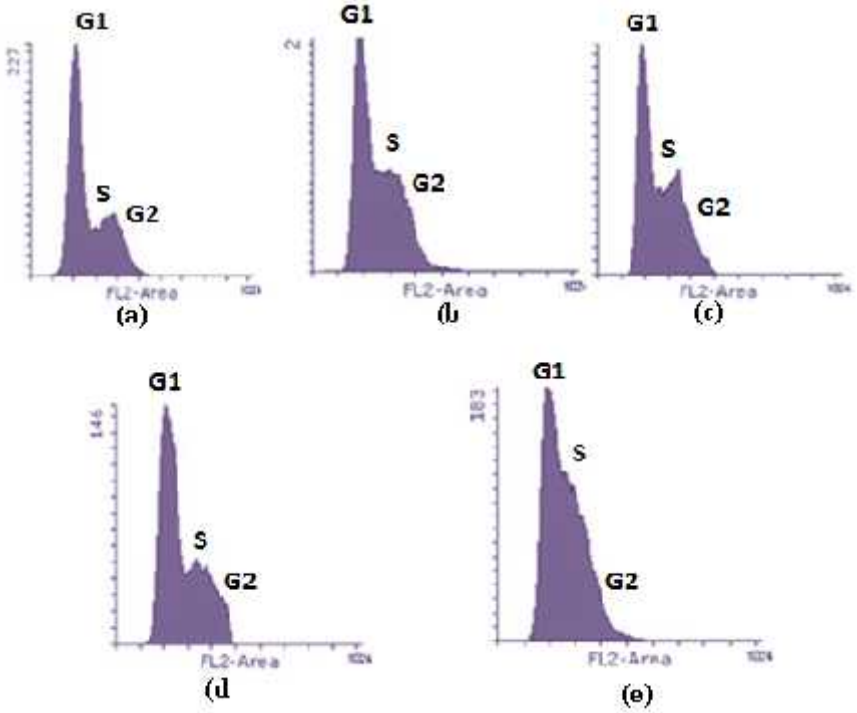


Fig. 5.The effect of treatment of the combination between the EAFB with cisplatin towards the growth of T47D cells.

As much as 8000 cells per well plates in 96 well plates were incubated for 24 h with or without treatment in DMEM Hi-glucose medium. Observation was done under an inverted microscope with a magnification of 100 times. (a) control; (b) 1/6 IC_{50} EAFB; (c) 1/6 IC_{50} Cisplatin; (d) combination ratio of 1/6 IC_{50}; (e) combination of ratio of 1/12 IC_{50}. The CI value of the combination between the EAFB with cisplatin shows synergistic effect (CI<1).

Observation of apoptosis caused by the combination of the EAFB with cisplatin

Apoptosis induction was observed to know the cell mechanism caused by the treatment of the EAFB, cisplatin, and the combination of the two to T47D breast cancer cells after 24 h of incubation. The combination between the EAFB with cisplatin used in the apoptosis observation used the concentration of 1/6 IC_{50}. The method used in this research was the Annexin V method that was detected by using flow cytometry to observe the apoptosis induction happened to the cells that was given treatment. Annexin V is a protein group that strongly binds negative charged cell membrane phospholipids. The cell death caused by apoptosis or necrosis can be differentiated by coloring using Propidium Iodide (PI) through intercalation with DNA [17]. The result of

apoptosis induction test by using flow cytometry (Fig. 6) and the percentage of cell death after the er apoptosis or necrosis is shown in Table 5.

Table 5: Death percentage after treatment

	Control	6 μg/mL EAFB	2.5 μM Cisplatin	6 μg/mL EAFB + 2.5 μM cisplatin
Initial Apoptosis (%) (Mean ±SD) *n* = 5 experiments	1.45 ± 0.13	1.52 ± 0.11	2.64 ± 0.76	10.92 ± 1.23
Final Apoptosis (%) (Mean ±SD) *n* = 5 experiments	1.63 ± 0.12	2.13 ± 0.54	2.53 ± 0.32	1.24 ± 0.98
Necrosis (%) (Mean ± SD) *n* = 5experiments	0.86 ± 0.08	0.98 ± 0.54	0.87 ± 0.43	1.62 ± 1.45
Total	3.94 ± 0.10	4.63 ± 0.45	6.04 ± 0.98	13.78 ± 1.89

SD: Standard deviation

The analysis of cell death percentage after the treatment of the EAFB, cisplatin, and the combination of the two (Table 5) shows that cells that were not given treatment exhibits living cells of 96.06% and cell death of 3.94%. The cells that were given single treatment with the EAFB shows cell death of 4.63%; treatment with cisplatin shows cell death of 6.04%; while the treatment with combination of the two shows cell death of 13.78%. This shows that the cell death percentage at the treatment with EAFB and cisplatin shows an increase of 7.74% combined compared to only cisplatin, so that the combination ratio of 1/6 IC_{50} induces apoptosis.

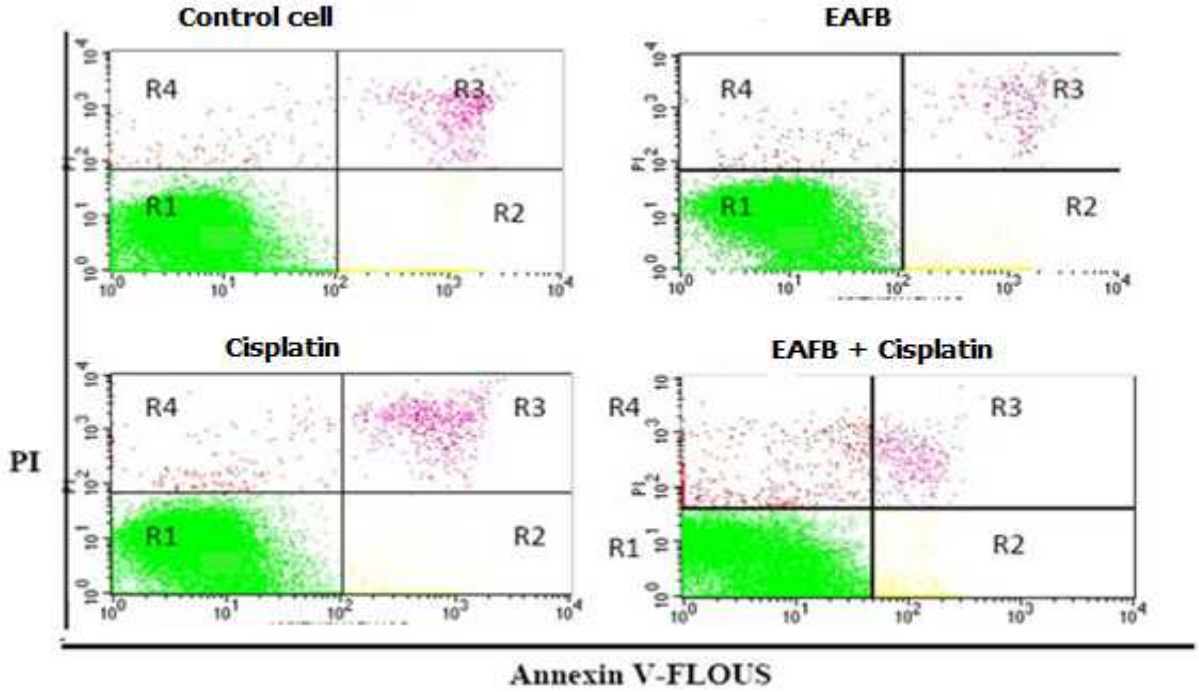


Fig. 6: The effect of apoptosis induction after the treatment of EAFB and cisplatin, and the combination of the two

The cells were planted with the density of 5×10^5 in 6 well plates and incubated for 24 h in DMEM High glucose medium either with or without treatment. The flow cytometry detection towards the cell death used

Annexin V FLUOS on T47D breast cancer cells after the treatment of 6 μg/mL EAFB and 2.5 μM cisplatin and the combination of the three. R1 quadrant shows life cells, while R2 shows initial apoptosis, R3 shows final apoptosis, R4 shows necrosis.

Discussion

T47D cell is a type of breast cancer cells that has the characteristic of caspase-3 wildtype, caspase-7 wildtype, positive ER/PR and p53 mutant [18]. The apoptosis induction that took place might has happened through the apoptosis mechanism that does not rely on p53. Cisplatin was reported to be able to induce downregulation Bcl-2 towards T47D breast cancer cells [19] and is able to create DNA crosslinks that results in damage in DNA that induces apoptosis [20]. Downregulation Bcl-2 (anti-apoptosis protein) will decrease the cell survivability and increases its sensitivity towards chemotherapeutic agent [21]. Result shows that the combination between the fraction and cisplatin increases apoptosis and undergoes cell cycle modulation at the S phase. The modulation on S phase or S arrestrenders cells unable to replicate and proliferate. The effects were also possible since the fraction contains several compounds, so the chance is high antagonistic effect to happen between the compounds,so it is needed to examine this further by analyzing the apoptosis induction of the compounds contained in the fraction. Further researches are needed to discover which proteins are involved to understand the molecular mechanism that bridges the synergism between the compounds in the fraction, p53 protein expression, Bcl-2, and NF- B in vitro on the T47D breast cancer cells.

Conclusion

The combination between 1/6 IC_{50} (6 μg/mL) EAFB and 2.5 μM cisplatin is able to increase the cytotoxic effect of cisplatin towards T47D, and has synergistic properties with the CI value of 0.58. The combination between the EAFB with cisplatin inflicts S arrest on T47D breast cancer cells. The combination between the EAFB with cisplatin increases apoptosis induction on T47D breast cancer cells.

Acknowledgements

The author would like to thank staff members of Medical Laboratory Gadjah Mada University.

Financial support and sponsorship: This research was funded by LPPM Ma Chung University, Indonesia in project "Ma Chung Research Grant 2017", No: 004/MACHUNG/LPPM-MRG-PRA//II/2017 ".

Conflict of Interests: The authors' declare no conflict of interest.

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