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Hedyotis corymbosa L. and *Sterculia quadrifida* R.Br Ethanolic Extract Enhances Cisplatin's Cytotoxicity on T47D Breast Cancer Cells Through Cell Cycle Modulation

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ABSTRACT

Cisplatin used as cancer drug with side effects and resistance. Ursolic acid is a compound found in *Hedyotis corymbosa* L., (HCoL) while 8-hydroxydehydrodunnione is found in *Sterculia quadrifida* R.Br (SQuR) which are proven to have the cytotoxic effect to cancer cells. This research aims to search the effect of cisplatin, ethanolic extract of HCoL and SQuR to 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 the T47D cell by using IC_{50} parameter. The combination was tested by determining their combination index (CI) and cell viability. The combination effect of 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 HCoL ethanolic extract as much as 0.21 µg/mL, SQuR ethanolic extract as much as 0.45 µg/mL, and cisplatin as much as 2.5 µM. The combination of HCoL ethanolic extract, SQuR ethanolic extract, and cisplatin results in phase S cell accumulation (27.73%) on T47D breast cancer cell and was able to induce apoptosis. The result proves that ethanolic extract of HCoL and SQuR can be developed as a co-chemotherapeutic agent to increase the effectivity of breast cancer treatment.

Key word: Cisplatin, Cytoxicity, *Hedyotis corymbosa* L., *Sterculia quadrifida* R.Br.

INTRODUCTION

Cancer was the significant mortality cause in the world with the number of 8.2 million deaths in 2012 and predicted increase continuously from 14.1 million cases in 2012 to 22.2 million in 2030 [1,2]. Breast cancer was the fifth major causes of total cancer deaths, 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].

Nowadays, chemotherapy is a strategy for treating breast cancer after surgery [3]. Chemotherapeutic agents usually show low selectivity properties due to antiproliferative properties against both cancer and normal cells [4]. Besides, 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 the cardiovascular system [6]. Trastuzumab was selective chemotherapeutic agents development 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.

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Cisplatin was the chemotherapeutic drug 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 in an incidence of drug resistance. The drug resistance associated with cisplatin occurred through changes in regulation on DNA repair, inhibition of apoptosis, cellular uptake, and drug efflux. Side effects due to cisplatin administration can occur when a high dose of cisplatin is given to reach more effective treatment [11]. Therefore, a research is needed to discover a more effective and selective breast cancer treatment method.

Indonesian medicinal plants, *Hedyotis corymbosa* L. (HCoL) and *Sterculia quadrifida* R.Br (SQuR), provides high potency to be developed as novel breast cancer chemopreventive agents. HCoL contains ursolic acid that is known to have anticancer activities with the mechanism of antiproliferative action and anti-angiogenesis. Angiogenesis was regulated by pro- and anti-angiogenic factors to make new blood vessels in cancer cells. Angiogenesis was highly needed by tumor cells to spread to another organ [12]. However, ursolic acid able to inhibit the activation of NF-κB and cytokines expression [13]. On the other hand, SQuR contains flavonoid, phenolic dan terpenoid that makes it was able to cure hepatitis, typhoid fever, and gastritis [14].

Administration of chemotherapeutic agents in a combination provide a synergistic effect, increase sensitivity of cancer cells and further reduce the dose of each chemotherapeutic agent to be used [15]. Based on the previous researches, the extracts obtained from HCoL SQuR were potential to combined with cisplatin as the chemotherapeutic agent for breast cancer treatment [16]. The combination between the extract of HCoL and SQuR with cisplatin was expected 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 HCoL and SQuR extracts with cisplatin showed cytotoxic activity to T47D cells through the cell cycle modulation and apoptosis induction.

EXPERIMENT

Chemicals and instrumentation

Materials used in this research were *Hedyotis corymbosa* L. (HCoL) and *Sterculia quadrifida* R.Br (SQuR) powder obtained from UPT Materi Materia Medica Batu, Malang. The concentrated extract from HCoL and SQuR were used as in vitro cytotoxic test samples. TLC Silica gel 60 F_{254} (Merck). The concentrated extract was diluted with dimethyl sulfoxide (DMSO) 0.1% in the medium. The chemotherapeutic agent used was cisplatin (Wako). T47D cell was grown in the high glucose DMEM (Dulbecco's Modified Eagle Media) culture (Gibco) contains 10% Fetal Bovine Serum (FBS) (v/v) (FBS Qualified, Gibco, Invitrogen USA), 5% Fungizone (v/v) (Gibco) and 1.5% penicillin-streptomycin (v/v) (Gibco, Invitrogen USA). The cell harvesting from Tissue Culture Dish (IWAKI) used 0.25% trypsin-EDTA (Gibco, Invitrogen Canada). Cytotoxicity test for both the cisplatin and the ethanolic extract of *Hedyotis corymbosa* L., the ethanolic extract of *Sterculia quadrifida* R.Br, and cisplatin mixture were done using MTT assay. The reagent used was 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, USA). MTT 5 mg/mL, diluted in phosphate buffer saline (PBS) pH 7.4. Working reagent was made by diluting the stock ten times using culture medium. PBS pH 7.4 was made by diluting 8 g NaCl; 0.2 g KCl; 0.2g KH_2PO_4; 1.15 g Na_2HPO_4 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). The instruments used are autoclave (AC-300AE, Tiyoda Manufacturing Co. Ltd), shaking incubator, TLC chamber, Laminar Air Flow cabinet (FARRco) and UV-Vis spectrophotometer (Shimadzu).

The instruments used in this research was autoclave (Hirayama HV 25 020585175, Hirayama Manufacturing Co., Japan), 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 mm^2, 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, lowa, USA), oven (Memmert), ELISA reader (Bio-Rad microplate reader Benchmark serial no. 11565, Japan), FACTScalibur flow cytometer.

Extraction

The flower of HCoL and the stem bark of SQuR were cleaned and washed with running water, then dried under 50°C for 4 days. The simplicial of the flower of HCoL and the stem bark of SQuR were grinded to powder. Maceration method was used to extract using 96% ethanol as solvent. About 250 g of the powder was 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. The precipitate was then re-macerated. After the extract thickened, it was deemed to be suitable for further test.

Identification of Chemical Compounds in Extract

Two milligrams of HCoL and SQuR extracts were weighed and diluted in 1 mL of ethanol. The diluted extract was spotted on TLC 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. TLC plate was sprayed with cerium sulfate and Dragendorff reagents. The plate dried briefly in the oven and the spot formed used to calculate the hRf values.

Cell Preparation and Harvesting

T47D cell in the cryogenic tube was taken from the liquid nitrogen tank and put into the LAF which is previously sprayed with alcohol and was waited until it melted. Then, the T47D cell was moved into a conical tube containing complete DMEM high glucose media. The conical tube containing cell suspension was centrifuged at speed 600 rpm for 5 minutes and removed the supernatant. Afterward, new media was added into the conical tube containing pellet and was suspended until it became homogeneous. The cell suspension grew in the Tissue Culture Dish (TCD) and incubated in the CO_2 incubator at of 37°C. The cell as observed under light microscope and then 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. Cells suspension transferred into the sterile conical tube. The number of cells was calculated using hemocytometer and cell counter. The cell suspension was made with the needed concentration. The single and combination cytotoxicity test used the cell density of 8×104 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

The test solutions stock of ethanolic extracts of HCoL and SQuR was made by diluting 5 mg of the extracts in 50 µL of DMSO. The solution stock of HCoL and SQuR ethanolic extracts 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 three combination treatment between extracts of HCoL and SQuR 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 extracts of HCoL and SQuR 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 8×10^3cells per well plates and were diluted with the culture medium, 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. Removed PBS and 100 µL/well plates of test solution were added. Cells incubated further for 24 h. After incubation, MTT reagent was added as much as 100 µL/well plates followed by 3-4 h of incubation at 37°C. Afterward, 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 for reading 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 with the combination of HCoL and SQuR extracts with the chemotherapeutic agent cisplatin in various combination ratios. Cytotoxicity test of HCoL and SQuR extracts 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 HCoL and SQuR extracts Extracts with Cisplatin

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

HCoL: *Hedyotis Corymbosa L* extract; SQuR: *Tinospora Crispa* extract; C: Cisplatin; CC: Cell Control; MC: Medium Control.

Cell Cycle Observation

Transferred 5×105 cells/well into 6 well plate each as much as 1000 µL, then incubated the cells until normal condition. HCoL and SquR extracts, as well as cisplatin, were added in concentration series for the treatment. The concentrations were based on the cell viability result obtained from the combination test. To the group treated with combination test, HCoL and SQuR extracts were added each 300 µL into the well plates, then 300 µLof cisplatin was added. Meanwhile, for the single treatment, 900 µL of HCoL and SQuR extracts or cisplatin in the well plates and the control cells, 900 µL of culture medium was added into well plates then incubated for 24 h. 1.5 mL medium were taken and transferred into the conicals at the and of process. At the well plates which medium was taken, 500 µL of PBS was added into the well plates and transferred into the same conical with similar treatment. After this, transferred 200 µL of 0.25% trypsin-EDTA to harvested the cells and incubation for 3 min, followed by the addition of 1 mL culture medium and resuspension until the cells detached one by one as seen under the microscope. The harvested cells were transferred into the same conical. Then, the mixture in the conical was centrifuged with the speed of 2000 rpm for 5 min and the PBS was removed. The cells precipitate was washed with 500 µL of cold PBS and re-centrifuged at the speed of 2000 rpm for 5 min and the PBS was removed. Added Flow cytometry reagent and analyzed with FACS Calibur for cell cycle profile, the data analyzed using the flowing program.

Bcl-2 Protein Expression Assay

A number of T47D cells were incubated for 24 h in several concentrations of plant extracts and cisplatin combination. The cells were washed with cold PBS. Added to the cells as much as 500 µL lysis buffer (70 mM KCl, 250 mM sucrose, 0.25% Triton X-100 in the PBS containing protease inhibitor), then incubated for 10 minutes. Centrifuged cell lysate at 20,000 rpm for 20 minutes. The supernatant that contains proteins (50 µg) were separated with 15% SDS-PAGE so a band of protein can be taken. The bands were transferred onto nitrocellulose membrane. Then, a number of 5% PBS containing 0.2% Tween-20 were added into the membrane so it can be incubated with polyclonal antibodies of rabbit (Bak, Bid dan Bcl-xL) and the monoclonal antibody of mice (Bax). X-Ray film used for visualized protein bands. Densitometer using to determinate the band's density. The results were analyzed with image software.

Statistical Analyses

The absorbance of cells was converted to the percentage of living cells. Synergistic cytotoxic determined by calculating the combination index (CI combinatorial methods or index) by Chou (Reynolds and Maurer, 2005) 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 upper left (UP), lower left (LL), upper right (UP), lower right (LR). 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. Apoptosis is known to compare the effects of single compounds and combination treatment with control cells. Flow cytometry data distribution of the percentage of cells in each phase G1, S and G2/ M. The inhibition of the cell cycle process determined by comparing the treatment effect of the test solution with control cells.

RESULT AND DISCUSSION

Identification of Chemical Compounds in Extract Test

The identification of chemical compounds in HCoL and SQuR extracts was performed to ensure that both extracts contain alkaloids and terpenoids which are scientifically proven to have cytotoxic properties.

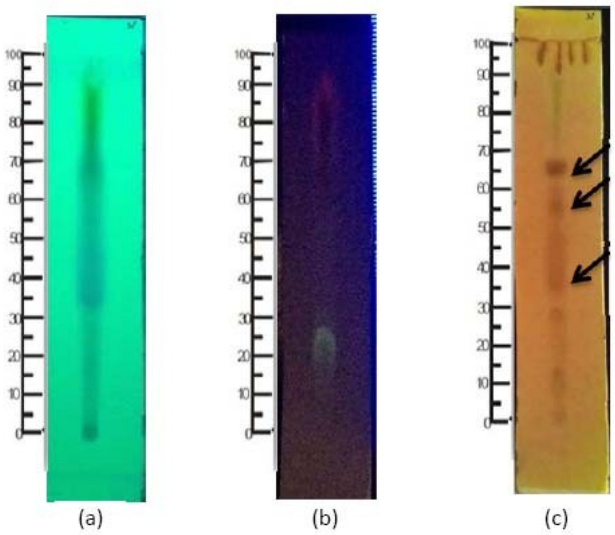


Figure 1. The result of (a) UV 254 Test, (b) UV 366 Test, and (c) Dragendorff Test on SQuR

The test result on SQuR stem extract (Figure 1.) shows no spot on UV 254 and UV 366 test so it needs color detection test by using Dragendorff reagent. After Dragendorff reagent was sprayed onto the plates, there were 3 spots with hRf of 66, 55, and 35 in reddish orange. The spots indicate 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 the heavy metal in the Dragendorff will create a bond with a lone pair electron in the N atom of alkaloids [17].

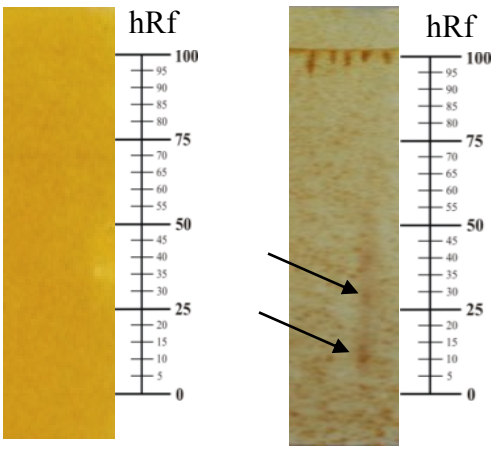


Figure 2. The result of Dragendorff Test (a), Cerium Sulfate Test (b) on HCoL extract

The test result on HCoL extract (Figure 2) after being sprayed with cerium sulfate reagent shows 2 brown spots with the hRf value of 16 and 25 shows carbon atom, while no spots being spotted after spraying with Dragendorff reagent indicates that HCoL extract doesn't contain alkaloids.

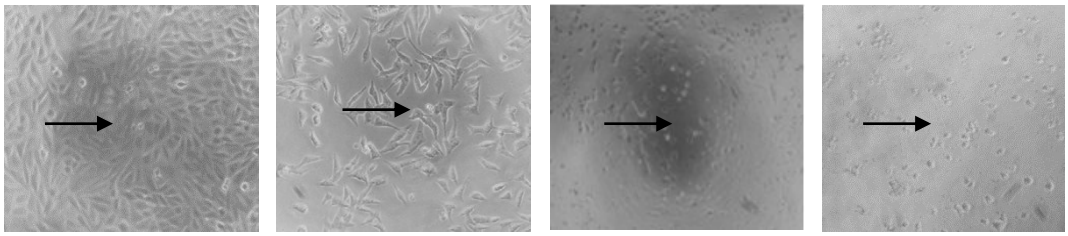
Single Cytotoxicity Test Result On the Ethanolic Extract of HCoL and SQuR with Cisplatin at The T47D Breast Cancer Cells

Cytotoxicity test was done to determine the potential of ethanolic extracts of HCoL and SQuR 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 ethanolic extract of HCoL, SQuR, and cisplatin was shown with the decrease of cell viability and morphological change on T47D breast cancer cells.

Table 2. IC_{50} Value of Ethanolic Extract of HCoL and SQuR and Cisplatin on T47D Cells

Sample	Linear Regression Equation	IC_{50} (Mean ± SD) *n* = 3 experiments
Ethanolic Extract of HCoL	y= 44.55x – 89.64	1.24 ± 1.26 µg/mL
Ethanolic Extract of SQuR	y= -45.93 + 46.76	2.69 ± 0.54 µg/mL
Cisplatin	y= -74.74 + 87.96	15.82 ± 0.75 µM

SD: Standard deviation, IC_{50} =Inhibition concentration 50%



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

Figure 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. The observation was done under an inverted microscope with a magnification of 100 times. (a) cell control; (b) 25 µg/mL of HCoL; (c) 50 µg/mL SQuR, (d) 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 ethanolic extract of HCoL (Figure 3b), ethanolic extract of SQuR (Figure 3c), and cisplatin (Figure 3d) shows a decrease the number of living cells compared to the control (Figure 3a). The cells look round and fragmented which indicates a change in cell morphology, but it was yet to know 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 Ethanolic Extract of HCoL and SQuR and Cisplatin on T47D Breast Cancer Cells

The cytotoxicity test on the combination was done to analyze the effect of adding the ethanolic extract of HCoL and SQuR 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, therefore it was done by reducing the concentration of cisplatin as the chemotherapeutic agent. The combination index value used to see the effect of the combination between the ethanolic extract of HCoL and SQuR and cisplatin. The efficacy classifications produced were synergistic, additive, or antagonistic. The cytotoxicity test for the combination was done using MTT assay.

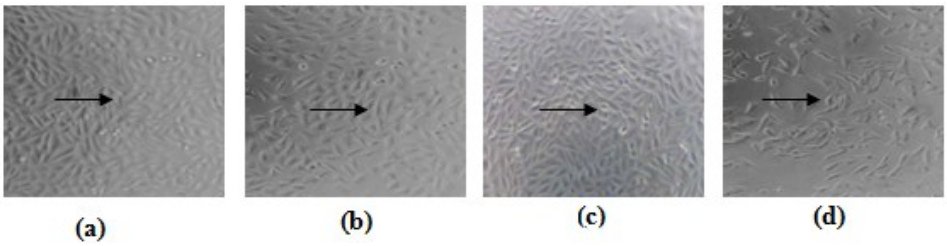


Figure 4. The Effect of Treatment of the Combination Between the Ethanolic Extract of HCoL and SQuR with Cisplatin Towards the Growth of T47D Cells.

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. The observation was done under an inverted microscope with a magnification of 100 times. (a) cell 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 ethanolic extract of HCoL andSQuR with cisplatin shows synergistic effect (CI<1).

The concentration series of the combination for the ethanolic extract of HCoL was 0.5, 1, 2 µg/mL sequentially, the ethanolic extract of SQuR 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 of the ethanolic extract of HCoL and SQuR with cisplatin shows shrinkage and cell morphology changes (Figure4a-d). Combination ethanolic extract of HCoL and SQuR with cisplatin at the concentration ratio of 1/12, 1/6, and 1/3 resulted in the CI value not more than 1.00 (Table 3), so it proved that these combinations exhibited a synergistic effect.

Table 3. The Combination Index (CI) Value of The Combination of Cisplatin with The Ethanolic Extract of HCoL and SQuR on T47D Cells

Concentration Ratio	**Cell Viability (%) (Mean ± SD)** ***n* = 5 experiments**	**CI**
1/12 IC_{50}	49.34 ± 0.56	0.78
1/6 IC_{50}	40.22 ± 0.88	0.83
1/3 IC_{50}	31.45 ± 0.97	0.56

SD: Standard deviation, CI=Combination Index

The Modulation of Cell Cycle from The Combination of the Ethanolic Extract of HCoL and SQuR 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 does. Flow cytometry method was performed to observe the modulation of cell cycle and the primary targets in inhibiting the proliferation of cancer. The phase of cell recycles

(G1, S, and G2/M) able to detect with flow cytometry. Propidium iodide was used to color each phase since it has the ability to interact with DNA [18]. The observation of cell cycle profiles was done at the 24th h. Flow cytometry analysis using the flowing program is shown in Picture 8 and the detailed distribution percentage of the cell cycle is shown in Table 4. The cell control undergoes a cell distribution in G1, S, and G2/M phase. The result shows that the ethanolic extract of HCoL has the accumulation of cells in the S phase, while the ethanolic extract of SQuR has the accumulation of cells in the S and G2/M phase. Cisplatin results have accumulation of cells in the S phase.

The combination of the three has accumulation of cells in the S phase compared to the cell control. The result of combination treatment, the percentage of cell cycle distribution on the S phase was 27.73% higher than the treatment of only cisplatin with 12.76%. The cell accumulation on the S phase in three combinations showed an increase from 10.11% to 27.73% compared to the cells without treatment (control cells). The cell accumulation was caused by cell cycle arrest.

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	46.23 ± 1.22	10.11 ± 1.45	21.54 ± 0.88
HCoL Ethanolic Extract (0.21µg/mL)	41.22 ± 0.82	15.67 ± 0.99	17.42 ± 0.93
SQuR Ethanolic Extract (0.45 µg/mL)	37.65 ± 0.60	12.22 ± 0.64	22.43 ± 0.43
Cisplatin (2.5 µM)	44.42 ± 0.86	12.76 ± 0.87	27.67 ± 0.65
0.21 µg/mL of Ethanolic Extract of HCoL+ 0.45 µg/mL of Ethanolic Extract of SQuR+ 2.5 µM of Cisplatin	22.52 ± 0.65	27.73 ± 0.97	13.33 ± 0.39

SD: Standard deviation

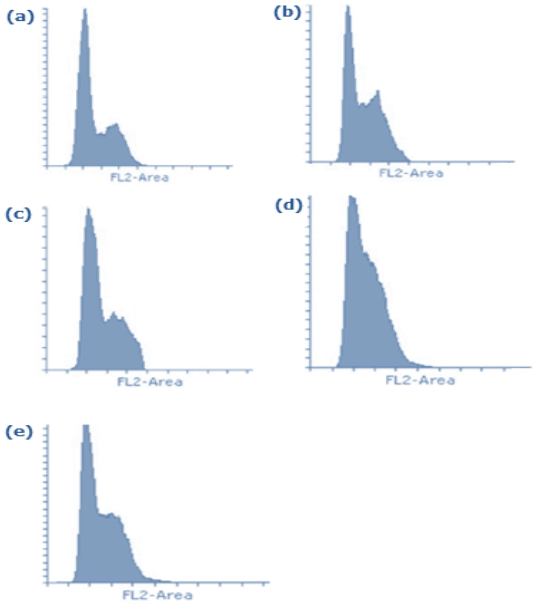


Figure 5. Cell Cycle Distribution Detection Using Flow cytometry.
(a) Cell Control; (b) 1/6 IC50 HCoL; (c) 1/6 IC50 SQuR; (d) 1/6 IC50 Cisplatin; (e) 1/6 IC50 HCoL - 1/6 SQuR -1/6 Cisplatin.

Observation of Apoptosis Caused by The Combination of the Ethanolic Extract of HCoL and SQuR with Cisplatin

Apoptosis induction was observed to know the cell mechanism caused by the treatment of the ethanolic extract of SQuR, the ethanolic extract of HCoL, cisplatin, and the combination of the three to T47D breast cancer cells after 24 h of incubation. The combination between the ethanolic extract of HCoL and SQuR 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 were given treatment. Annexin V is a protein group that strongly binds negatively charged cell membrane phospholipids. Propidium Iodide can be differentiated the cell death caused by apoptosis or necrosis [19]. The result of apoptosis induction using flow cytometry (Figure 6) and the percentage of cell death after the treatment of the combination of three was caused by apoptosis or necrosis is shown in Table 5.

Table 5. Death Percentage After Treatment

	Without treatment	**0.21 µg/mL Ethanolic Extract of *HCoL***	**0.45 µg/mL Ethanolic Extract of *SQuR***	**2.5 µM Cisplatin**	**0.21µg/mL Ethanolic Extract of *HCoL* + 0.45 µg/mL Ethanolic Extract of *SQuR* + 2.5 µM Cisplatin**
Initial Apoptosis (%) (Mean ±SD) *n* = 5 experiments	2.33 ± 0.13	2.25 ± 0.20	2.62 ± 0.11	2.54 ± 0.76	12.92 ± 1.23
Final Apoptosis (%) (Mean ±SD) *n* = 5 experiments	1.33 ± 0.12	2.98 ± 0.12	2.88 ± 0.54	2.88 ± 0.32	2.91 ± 0.98
Necrosis (%) (Mean SD) *n* = 5 experiments	0.67 ± 0.08	1.34 ± 0.54	1.89 ± 0.54	1.11 ± 0.43	1.98 ± 1.45
Total	4.33 ± 0.10	6.57 ± 0.23	7.39 ± 0.45	6.53 ± 0.98	17.81 ± 1.89

SD: Standard deviation

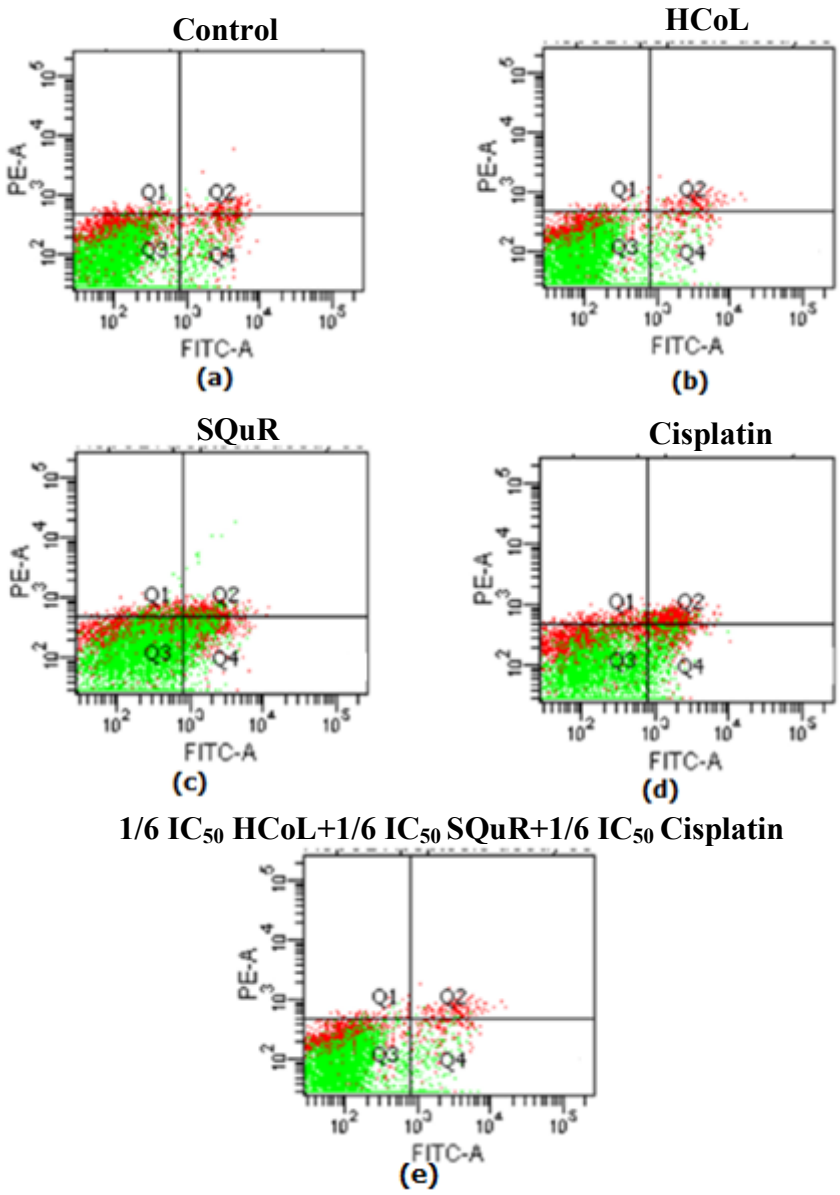


Figure 6. The Effect of Apoptosis Induction After the Treatment of Ethanolic Extract of HCoL, Ethanolic Extract of SQuR, Cisplatin, and The Combination of the Three.

The analysis of cell death percentage after the treatment of the ethanolic extract of HCoL, SQuR, cisplatin, and the combination of the three (Table 5) shows that cells that were not given treatment exhibit living cells of 95.67% and cell death of 4.33%. The cells that were given single treatment with the ethanolic extract HCoL shows cell death of 6.57%; treatment with the ethanolic extract of SQuR shows cell death of 7.39%; treatment with cisplatin shows cell death of 6.53%; while the treatment with combination of the three shows cell death of 17.81%. This indicates that the cell death percentage at the treatment with the ethanolic extract of HCol, SQuR, and cisplatin shows an increase of 11.28% combined compared to only cisplatin, therefore, the combined ratio of 1/6 IC_{50} induces apoptosis.

T47D cell is a type of breast cancer cells that have the characteristic of caspase-3 wildtype, caspase-7 wildtype, positive ER/PR and p53 mutant [19]. The apoptosis induction that took place might have happened through the apoptosis mechanism that does not rely on

p53. Bcl-2 protein family has 3 main protein types. They are pro-apoptosis proteins (contain multi-domain, such as Bax and Bak); anti-apoptosis proteins (contain multi-domain, such as Mcl-1, Bcl-2, and Bcl-xL); and pro-apoptosis protein containing BH3 domain (such as Bid, Bad, Bim, Noxa, and Puma) [21].

Bcl-2 protein family has the double role in apoptosis phenomenon that is mediated by mitochondrial pathway [21]. Bid (BH3) is a molecule that is linked to both extrinsic and intrinsic pathway. The molecule will be proteolyzed by caspase-8 from the extrinsic pathway and be stimulated from the mitochondrial pathway.

The apoptosis on T47D cells that had been incubated with the combination of HCoL, SQuR, and cisplatin shows an increasing in pro-apoptosis protein (Bax dan Bak) expression level; where BH3 (Bid) protein, being cleaved faster (forming either Bid or tBid). Bid protein will be proteolyzed by caspase-8 through extrinsic receptor pathway, while tBid will be activated by the other pro-apoptosis proteins through intrinsic pathway. This indicates that the combination of HCoL, SQuR, and cisplatin can induce apoptosis on T47D cell through both intrinsic (Bax, Bak) and extrinsic (Bid) pathway. The decreasing on the expression of anti-apoptosis protein (Bcl-xL) was being confirmed by the intrinsic pathway.

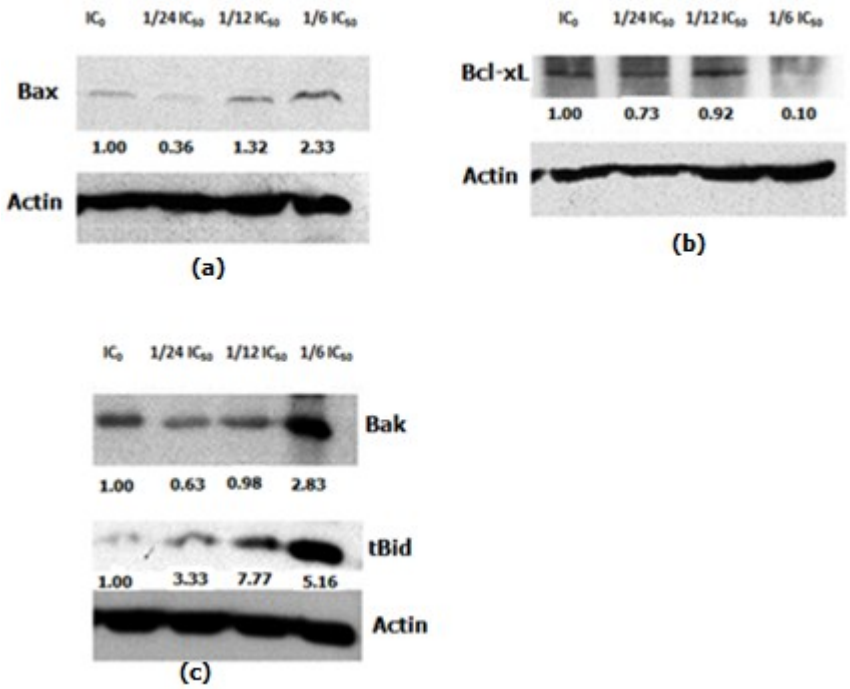


Figure 7. The Effect of Apoptosis Induction After the Treatment of Ethanolic Extract of HCoL, Ethanolic Extract of SQuR, Cisplatin, and The Combination of the Three on Anti-apoptosis and Pro-apoptosis Protein. (a) Effect on Bax and actin, (b) Effect on Bcl-xL and Actin, (c) Effect on Bak, tBid, and actin.

CONCLUSION

The combination between 1/6 IC_{50} (0.21µg/mL) of the ethanolic extract of HCoL and 1/6 IC_{50} (0.45 µg/mL) of the ethanolic extract of SQuR, and 2.5 µM cisplatin was able to enhance the cytotoxic effect of cisplatin towards T47D, and has synergistic properties with the CI value of 0.83. The combination of the ethanolic extract of HCoL and SQuR with cisplatin inflicts S arrest on T47D breast cancer cells. The combination between the ethanolic

extract of HCoL and SQuR with cisplatin increases apoptosis induction in T47D breast cancer cells by stimulating pro-apoptosis protein (Bax, Bak, tBid) and inhibiting anti-apoptosis protein expressions.

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