COMBINATION OF HEDYOTIS CORYMBOSA L. AND TINOSPORA CRISPA ETHANOLIC EXTRACT INCREASE CISPLATIN CYTOTOXICITY ON T47D BREAST CANCER CELLS

ROLLANDO ROLLANDO*
Program of Pharmacy, Faculty of Science and Technology, Ma Chung University, Malang, East Java, Indonesia. Email: ro.llando@machung.ac.id
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ABSTRACT

Objective: Ursolic acid was a compound found in *Hedyotis corymbosa* L. (HCoL) while berberine found in *Tinospora crispa* (TCa) which are proven to have cytotoxic effect to cancer cells. This research aims to review the effect of cisplatin, ethanolic extract of HCoL and TCa to the sensitivity increase on breast cancer cells, which will be confirmed through apoptosis induction and cell cycle modulation.

Methods: The cytotoxic effect was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on T47D cell using the IC₅₀ parameter. The combination was tested by determining their combination index (CI) and cell viability. The combination effect of apoptosis induction and cell cycle modulation was observed using flow cytometry method.

Results: The cytotoxic test result of the combination shows CI value of below 1 at the concentration of HCoL ethanolic extract as much as 1 µg/mL, TCa ethanolic extract as much as 6 µg/mL, and cisplatin as much as 2.5 µM. The combination of HCoL, ethanolic extract, TCa ethanolic extract, and cisplatin results in phase S cell accumulation (29.98%) on breast cancer cell T47D and was able to induce apoptosis.

Conclusion: The result proves that ethanolic extract of HCoL and TCa can be developed as a cochemotherapeutic agent with cisplatin to increase the effectivity of breast cancer treatment.

Keywords: Cisplatin, Cytotoxicity, *Hedyotis corymbosa* L., *Tinospora crispa*.

INTRODUCTION

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 [1,2]. Chemotherapy was 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 through several molecular changes [5], and harmful side effects on the 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 conventional chemotherapeutic agent still widely used due to economical consideration.

Cisplatin was chemotherapeutic agent used in breast cancer therapy as monotherapy or in a combination [9]. Cisplatin induces side effects such as neurotoxicity, nephrotoxicity, and bone marrow suppression [10]. Besides that, usage of cisplatin as a chemotherapeutic agent has an incidence of drug resistance. The drug resistance associated with cisplatin was 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 high dose of cisplatin was given to reach more effective treatment [11]. Therefore, researches were needed to discover more effective and selective breast cancer treatment method.

*Hedyotis corymbosa* L. (HCoL) and *Tinospora crispa* (TCa) provide high potency to be developed as novel breast cancer chemopreventive agents. HCoL contains ursolic acid that known to have anticancer activities with the mechanism antiproliferative action and anti-angiogenesis. Angiogenesis was physiological process in which new blood vessels from pre-existing vessels and was regulated by pro- and anti-angiogenic factors. Angiogenesis was highly needed by tumor cells to spread to another organ or metastatic [12]. Ursolic acid was able to inhibit the regulation of pro-inflammatory cytokines expression through the inhibition of NF-κB activation and antitumor [13]. In the other hand, TCa contains diterpenoid compounds that exhibit cytotoxic activities in human prostate cancer [14].

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

METHODS

Materials

Materials used in this research are HCoL and TCa powder obtained from UPT Materi Materia Medica Batu, Malang, Dimethyl sulfoxide (wako chemical USA), Cisplatin (wako chemical USA), DMEM (Gibco, Invitrogen USA), Fetal Bovine Serum (Gibco, Invitrogen USA), 1.5% penicillin-streptomycin (v/v) (Gibco, Invitrogen USA), 0.5% Fungizone (v/v) (Gibco, Invitrogen USA). Tissue culture dish (TCD) (IWAKI), Trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, Invitrogen Canada), reagent used was 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Invitrogen Canada), reagent used was 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Invitrogen Canada).
2.5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA), sodium dodecyl sulfate (Merck-Schuchardt, Germany), and propidium iodide (PI) solution in phosphate-buffered saline (PBS) which contains 1 mg/ml (minimum 95% high-performance liquid chromatography), Sigma-Aldrich Co, St Louis, MO, 63178, triton X-100 for GC, (E. Merck, 64271, Darmstadt, Germany), and annexin V-FLOUS Apoptosis Detection Kit (Roche, USA).

Instruments
The instruments used in this research were autolocate (Hinayama HV 25 020585175, Hirayama Manufacturing Co., Japan), liquid nitrogen, Labconco purifier Glass II biosafety cabinet (Delta Series, Labconco Corporation, Missouri, USA), CO2 incubator (Heraeus), inverted microscope (Nikon, Eclipse, TE 2000-U), hemocytometer (Neubauer improved 0.100 mm Tiefen Depth Profondeur 0.0025 mm2, Germany), digital camera (Sony), centrifuge (Sigma 203, R.Braun Biotech International), digital scale (Mettler Toledo, AG204 Delta Range), stirrer (Nuova, Thermolyne, mixer (Maxi Mix II, Thermolyne type 37600 mixer, Iowa, USA), oven (Memmert), and ELISA reader (Bio-Rad microplate reader Benchmark serial no. 11 565, Japan), FACTScalibur flow cytometer.

Extraction
The flower of HCoL and the stem of TCa were cleaned and washed with running water, then dried under 50°C for 4 days. The flower of HCoL and the stem of TCa were grinded to form a powder. Maceration method was used to extract using 96% ethanol as solvent. About 250 g of the powder were weighed and diluted in 1 L of ethanol and macerated for 1 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, it was deemed to be suitable for further test.

Identification chemical compounds
1 mg of HCoL and TCa extracts were weighed and diluted in 1 mL of ethanol. The diluted extract is then spotted on a plate. The mobile phase used was chlorofom:ethanol 9:1 (v/v). Then, a chamber to place the mobile phase was prepared and thin layer chromatography (TLC) 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 sulfate and Dragendorff reagents. The plate is then dried briefly in the oven and the spot formed is used to calculate the hRf value.

Preparation of test solution
The solution stock of HCoL and TCa ethanolic extracts were diluted using culture medium to the concentration of 1, 10, 25, 50, 100, and 200 µg/mL to be used as the stationary phase and the mixture of chloroform:methanol (9:1, v/v) as the mobile phase used was chloroform:methanol (9:1, v/v) as the mobile phase used to extract using 96% ethanol as solvent. About 250 g of the powder were weighed and diluted in 1 L of ethanol and macerated for 1 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, it was deemed to be suitable for further test.

Identification of chemical compounds in extract test
The extract obtained was then evaporated and the ethanolic extracts were diluted using culture medium to the concentration of 1, 10, 25, 50, 100, and 200 µg/mL to be used as the single cytotoxicity test solution. Cisplatin test solution was added with 300 µL of HCoL and TCa extracts was added with 300 µL of cisplatin with a concentration series, while for the single treatment, 900 µL of HCoL and TCa extracts and cisplatin was added into the well plates following a concentration series. For the control cells, 900 µL of culture medium was added to the well plates. After treatments, all cells were incubated for 24 h. The cell precipitate formed was placed in conical tubes with aluminum foil as its cover and was diluted with annexin V-FLOUS buffer kit by 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
IC50, calculated with probit analysis method. Data obtained absorbance was converted to a percentage of living cells. Synergistic cytotoxic determined by calculating the combination index (CI) (CI combinatorial methods or index) and drug reduction index using software Compusyn (www.combosyn.com) and the resulting isobologram. Flow cytometry data show the percentage of cells contained in four quadrants, namely lower left, lower right, upper left, and upper right. The quadrants to analyze the distribution of cells percentage in G1, S and G2/M phase. 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 extract test
The test result on TCa stem extract (Fig. 1) shows one brown spot after being sprayed with cerium sulfate reagent that shows hRf 5 indicates that TCa stem 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 (KI) 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 [16].

Silica gel 60 F254 as the stationary phase and the mixture of chloroform:methanol (9:1, v/v) as the mobile phase
The test result on HCoL extract (Fig. 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 does not contain alkaloids.

Table 1: Ratio of concentration used in the combination of HCoL and TCa extracts with cisplatin

| HCoLa (1/12 IC50) | TCa (1/12 IC50) | C (1/12 IC50) | HCoL (1/12 IC50) | TCa (1/12 IC50) | C (1/12 IC50) | HCoL (1/6 IC50) | TCa (1/6 IC50) | C (1/6 IC50) | HCoL (1/3 IC50) | TCa (1/3 IC50) | C (1/3 IC50) |
|------------------|-----------------|--------------|------------------|-----------------|--------------|------------------|-----------------|--------------|------------------|-----------------|--------------|
| CC               | CC              | MC           | CC               | CC              | MC           |

HCoL: Hedyotis Corymbosa L extract, TCa: Taxispora Crispa extract, C: Cisplatin, CC: Control cell, MC: Medium control
Silica gel 60 F254 as the stationary phase and the mixture of chloroform: methanol (9:1, v/v) as the mobile phase.

**Single cytotoxicity result on T47D cell lines**

Cytotoxicity test was done to determine the potential of ethanolic extracts of HCoL and TCa with cisplatin in inhibiting T47D breast cancer cell. Before the cytotoxicity test was done on the combination of the three, the individual IC50 value was calculated to determine the concentration suitable for each component based on their IC50 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, TCa, and cisplatin was shown with the decrease of cell viability and morphological change on T47D breast cancer cells (Table 2).

The concentration series of the combination for the ethanolic extract of HCol was 0.5, 1, 2, µg/mL sequentially, the ethanolic extract of TCa was 2.5, 5, and 10 µg/mL, and cisplatin was 1.25, 2.5, and 5 µM. The cell morphology changes of T47D cells caused by the combination of the ethanolic extract of HCoL and TCa with cisplatin show shrinkage and cell morphology changes (Fig. 4a-d). Combination ethanolic extract of HCol and TCa with cisplatin show synergistic effect (p<0.05).

**Combination cytotoxicity test result on T47D breast cancer cell**

The cytotoxicity test on the combination was done to analyze the effect of adding the ethanolic extract of HCol and TCa into the combination with cisplatin on T47D breast cancer cells. The IC50 value was calculated from the linear regression between log of the concentration and percentage viability with the confidence value of 95% (p<0.05). The concentration ratio used for the combination was 1/12, 1/6, and 1/3 of the IC50 value. The concentrations were of a lower concentration compared to IC50. 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 CI value was the parameter used to see the effect of the combination between the ethanolic extract of HCol and TCa and cisplatin. The efficacy classifications produced were synergistic, additive, or antagonistic. The cytotoxicity test for the combination was done using MTT assay.

Table 2: IC50 value of ethanolic extract of HCol and TCa and cisplatin on T47D cells

| Sample                  | IC50 (mean±SD)   |
|-------------------------|------------------|
| Ethanolic extract of HCol| 4.48±1.36 µg/mL  |
| Ethanolic extract of TCa| 13.15±0.45 µg/mL |
| Cisplatin               | 14.22±0.34 µM    |

n: 5, SD: Standard deviation, IC50: Inhibition concentration 50%. HCol: Hedyotis corymbosa L, TCa: Tinospora crispa

**The modulation of cell cycle from the combination on T47D breast cancer cells**

The DNA synthesis on the cancer cells goes through a cell cycle, as the one on normal cells does. One of the main targets in inhibiting the proliferation of cancer cells is by the modulation of the 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. PI was used to color each phase since it has the ability of interacting with DNA [17]. The observation of cell cycle profiles was done at the 24th h. Flow cytometry analysis using the flowing program is shown in Fig. 5 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. Research shows that the ethanolic extract of HCoL results in the accumulation of cells in the S phase, while the ethanolic extract of TCa 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.

**Apoptosis effect from the combination**

Apoptosis induction was observed to know the cell mechanism caused by the treatment of the ethanolic extract of TCa, the ethanolic extract of HCoL, cisplatin, and the combination of the three to T47D breast cancer cells after 24 h of incubation. Combination the ethanolic extract of HCoL, TCa, and cisplatin use 1/6 IC50 concentration for apoptosis observation. The method used in this research was the Annexin V method that was detected 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 PI through intercalation with DNA18. The result of apoptosis induction test using flow cytometry (Fig. 6) and the percentage of cell death after the treatment of the combination between the ethanolic extract of HCoL and TCa with cisplatin which is caused by either apoptosis or necrosis is shown in Table 5.

The analysis of cell death percentage after the treatment of the ethanolic extract of HCoL, TCa, cisplatin, and the combination of the three (Table 5) shows that cells that were not given treatment exhibit living cells of 96.06% and cell death of 3.94%. The cells that were given single treatment with the ethanolic extract HCoL show cell death of 3.61%; treatment with the ethanolic extract of TCa shows cell death of 4.63%; and treatment with cisplatin show cell death of 6.04%; while the treatment with a combination of the three shows cell death of 13.78%. This shows that the cell death percentage at the treatment with the ethanolic extract of HCoL, TCa, and cisplatin shows an increase of 7.74% combined compared to only cisplatin so that the combination ratio of 1/6 IC50 induces apoptosis.

The flow cytometry detection toward the cell death used Annexin V FLUOS on T47D breast cancer cells after the treatment of 1 µg/mL ethanolic extract of HCoL, 5 µg/mL ethanolic extract of TCa, 2.5 µM cisplatin, and the combination of the three. R1 quadrant shows living cells, while R2 shows initial apoptosis, R3 shows final apoptosis, and R4 shows necrosis.

**DISCUSSION**

T47D cell is a type of breast cancer cells that have the characteristic of caspase-3 wild-type, caspase-7 wild-type, positive ER/PR, and p53 mutant [16]. 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 toward T47D breast cancer cells [17] and is able to create DNA crosslinks that result in damage in DNA that induces apoptosis [18]. Downregulation Bcl-2 (anti-apoptosis protein) will decrease the cell survivability and

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**Table 3: The CI value of the combination between cisplatin with the ethanolic extract of HCoL and TCa on T47D cells**

| Concentration ratio | Cell viability (%) (mean±SD) | CI |
|---------------------|------------------------------|----|
| 1/12 IC50          | 52.86±0.56                   | 0.43|
| 1/6 IC50           | 45.76±0.88                   | 0.58|
| 1/3 IC50           | 39.87±0.97                   | 0.86|

n: 5, SD: Standard deviation, CI: Combination Index. HCoL: Hedysoty corymbosa L, TCa: Tinospora crispa

**Table 4: Percentage of cell cycle distribution after treatment**

| Sample                                | G1 phase (%) (mean±SD) | S phase (%) (mean±SD) | G2/M phase (%) (mean±SD) |
|---------------------------------------|------------------------|-----------------------|--------------------------|
| Control                               | 48.98±1.22             | 11.62±1.45            | 24.68±0.88               |
| HCoL ethanolic extract (1 µg/mL)      | 42.82±0.82             | 17.92±0.99            | 16.83±0.93               |
| TCa ethanolic extract (6 µg/mL)       | 34.83±0.60             | 13.82±0.64            | 24.03±0.43               |
| Cisplatin (2.5 µM)                    | 43.87±0.86             | 11.24±0.87            | 29.98±0.65               |
| 1 µg/mL of ethanolic extract of HCoL+6 µg/mL | 24.82±0.65             | 29.98±0.97            | 15.98±0.39               |
| of ethanolic extract of TCa+2.5 µM of cisplatin |                       |                       |                          |

n: 5, SD: Standard deviation. HCoL: Hedysoty corymbosa L, TCa: Tinospora crispa

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increases its sensitivity toward chemotherapeutic agent [19]. The result shows that the combination between the extracts and cisplatin increases apoptosis and undergoes cell cycle modulation at the S phase. The modulation on S phase or S arrest renders cells unable to replicate and proliferate. The effects were also possible since the extracts contain 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 ethanolic extract of HCoL and TCa.

In the G1 and S phases, cell regulation was inhibited by cyclin-dependent kinase inhibitors such as INK4 and CIP/KIP proteins and tumor suppressor genes, namely pRB and p53. In this study used T47D cells that have mutations p53 so that the anticancer mechanism of the combination was possible through a p53 independent pathway [20]. Independent pathway of p53 status and the occurrence of arrest possibilities because of the rapid decline in Gdc25A activity due to ubiquitination and events its degradation by the proteasome [21]. Loss of activity of Gdc25A phosphatase inhibited the activity of CDK2 kinase by inhibiting CDK2 dephosphorylation in threonine 14 and tyrosine 15 [22]. Phosphorylation of Cdc25A is mediated by ATM/ATR-Chk2/Chk1 cascade pathway. Degradation of Gdc25A phosphatase-dependent on ATM-Chk2 where inhibiting CDK2 will initiate cdc45 (replication checkpoint) which reduces the speed of DNA replication so that DNA replication stops and triggers S arrest [23]. The p38MAPK pathway also plays a role in the inhibition of cell cycle in phase S, which can occur through p53-independent [24]. The p38 protein can directly phosphorylate and stabilize p21 in vivo. The p38 protein may also phosphorylate and promote the degradation of Gdc25A which contributes to the termination of the cell cycle in phase S [25].

Further research needed to discover which proteins are involved to understand the molecular mechanism that bridges the synergism between the compounds in the extracts, p53 protein expression, Bcl-2, and NF-кB in vitro on the T47D breast cancer cells.

CONCLUSION

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

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AUTHORS’ CONTRIBUTIONS
Rollando Rollando participated in developing the research protocol, fieldwork supervision, data analysis, and drafting this manuscript.

CONFLICTS OF INTEREST
The authors declare that there are no conflicts of interest regarding the publication of this paper.

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