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Combination of cisplatin, *Hedyotis corymbosa* L. and *Tinospora crispa* extracts as a new therapy for breast cancer cells 4T1 through *in vitro* induction and cell cycle modulation**Rollando Rollando*, Muhammad Hilmi Afthoni***Department of Pharmacy, Faculty of Science and Technology, Ma Chung University, Malang
Jl. Villa Puncak Tidar No.1, Doro, Karangwidoro, Kec. Dau, Malang, East Java, Indonesia**Submitted: 07-09-2019**Reviewed: 21-11-2019**Accepted: 18-03-2020***ABSTRACT**

Cisplatin is a chemotherapeutic agent with varying side effects. Berberine compounds in *Tinospora crispa* and ursolic acids in *Hedyotis corymbosa* L are known to have high cytotoxic activities. This study was aimed to examine the effects of the combination of cisplatin, the ethanolic extract of *Hedyotis corymbosa* L.(EEHC), and the ethanolic extract of *Tinospora crispa* roots (EETC) on enhanced breast cancer cell sensitivity by apoptotic induction and cell cycle modulation. Cytotoxic effects were tested using the MTT assay in breast cancer cells, 4T1. The combination test of cisplatin and the extracts yielded combination index (CI) and cell viability, and its effects on the induction of apoptosis and cell cycle modulation were observed by the flow cytometry method. The results of the cytotoxic test showed CI of less than one for 1 mg/mL EEHC, 6 mg/mL EETC, and 1.68 mg/mL cisplatin. The EEHC-EETC-cisplatin combination accumulated cells in the S phase (29.98%) and induced apoptosis in 4T1 cells. It proves that EEHC and EETC can be developed as co-agents with cisplatin to improve the effectiveness of breast cancer chemotherapeutic treatment.

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

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INTRODUCTION

Cancer is one of the leading causes of death in the world. In 2008, it reportedly led to approximately 7.6 million deaths (around 13% of total mortality), and this figure is expected to increase to 13.1 million in 2030 (WHO, 2013). Breast cancer ranks first in the number of cases of cancer in women worldwide, with the incidence rate of 1,676,633 (IARC, 2012).

Chemotherapy is the widely proposed treatment for cancer. Chemotherapeutic agents generally have low selectivity because they are antiproliferative against cancer cells and healthy cells (Valeriote *et al.*, 2002). Also, several chemotherapeutic agents have a narrow therapeutic index and can lead to multiple drug resistance and adverse side effects (Minami *et al.*, 2010). Chemotherapy drugs that are mostly used in the treatment of breast cancer are cisplatin. Cisplatin, especially in high doses, can cause varying side effects (Florea and Busselberg, 2011), including neurotoxicity, nephrotoxicity (Milosavljevic *et al.*, 2010), and bone marrow suppression. Therefore, research focusing on finding active and selective methods to cure breast cancer becomes necessary.

Cancer cell treatment strategy is not a one-target but multiple-target mechanism, especially for cell proliferation and migration (Kemper *et al.*, 2014). 4T1 is an example of cancer cells in the metastatic phase that show migration and invasion (Kaur *et al.*, 2012).

Indonesia has various resources that can be potentially developed into chemotherapeutic agents against breast cancer, such as *Hedyotis corymbosa* L. and *Tinospora crispa*. *H. corymbosa* contains ursolic acid, which is known to possess antiproliferative and antiangiogenic activities, preventing the spread of cancer cells to other organs. Also, ursolic acid can inhibit the regulation of proinflammatory cytokines expression by inhibiting the activation of NF- κ B (Foo and Nolan, 1999). Berberine isolated from *Tinospora crispa* has cytotoxic activity against HCT-8 cells, NUGC Cells, and Hone-1 cells (Damu *et al.*, 2009).

Doses of chemotherapeutic agents can be reduced by combining them with compounds found in natural ingredients as an attempt to minimize adverse side effects while maintaining similar therapeutic properties against target cells (Zhao *et al.*, 2005). Moreover, Zhao *et al.* (2005) confirm that *H. corymbosa* and *T. crispa* extracts can be potentially developed in combination therapy with cisplatin for breast cancer treatment. This combination is expected to lower the required dose of cisplatin and, consequently, lessen its side effects. This study was conducted to assess the cytotoxic effects of the combination of the ethanolic extract of *H. corymbosa* (EEHC), the ethanolic extract of *T. crispa* (EETC), and cisplatin through cell cycle modulation and apoptosis induction.

MATERIALS AND METHOD

Materials

Materials used in this research were *Hedyotis corymbosa* L. and *Tinospora crispa* powder obtained from UPT Materi Materia Medica Batu, Malang.

Methods

Extraction of *H. corymbosa* roots and *T. crispa* leaves

H. corymbosa weighing 250 grams was macerated with 1 liter of 96% ethanol for one day. The liquid was then evaporated with a solvent to create a thick consistency (Rollando, 2018). This procedure was also applied to *T. crispa*.

Identification of chemical compounds in extracts

Two mg of both extracts were dissolved in 1 mL of ethanol. The extracts were spotted on TLC plates and then inserted into a chamber containing chloroform-ethanol 9: 1 as the mobile phase. Once the mobile phase reached the predefined mark, the plates were removed, sprayed with

Cerium (IV) Sulfate hydrate and Dragendorff's reagent, and dried in an oven. The band formed was then observed to calculate the HRF value.

Cell preparation and harvesting

The 4T1 cells were transferred to a conical tube containing high-glucose DMEM and centrifuged at 600 rpm for 5 minutes. After the supernatant was discarded, new media was added to the flask, and the cells were suspended until homogeneous. Cell suspensions were grown in Tissue Culture Dish (TCD) and incubated in a CO₂ incubator at 37°C. Cells were observed under a microscope and incubated in a 5% CO₂ incubator. After reaching ±80% confluence, they were harvested by removing the media. Then, the cells were washed with 3 mL of PBS 2 times, added with 0.25% trypsin-EDTA, and incubated for 1 minute in a CO₂ incubator.

A total of 3 mL of the media were added into TCD that contained the cells. Then, the cells were resuspended on the walls and at the bottom of TCD using a micropipette to prevent cell clumping. The cell suspension was transferred to a new sterile conical tube, and it was enumerated using a hemocytometer and cell counter. The cells were then formed into a cell suspension at predefined concentrations. The cytotoxic tests of single and combination solutions were performed at a density of 8x10⁴ cells.

A stock solution of each extract was diluted with the culture medium to obtain concentrations of 1, 10, 25, 50, 75, 100, and 200 mg/mL. These solutions were used in single cytotoxic tests. Meanwhile, the cisplatin test solution was diluted with the culture medium at concentrations of 0.5, 1, 2, 4, and 8 µg/mL (Rollando, 2018).

Cytotoxic tests of single and combination treatments using MTT assay

Cells were harvested, diluted with the culture medium, implanted into a 96-well microplate (with a volume of 100 µL/wells), and incubated for 24 hours in a 5% CO₂ incubator. Before the treatment, the media in the plate was removed and washed with 1x PBS (as many as 100 µL/pitting). Afterward, the PBS was removed, and the cells were given a test solution of 100 µL/pitting (Rollando, 2018).

After 24 hours of incubation, the cells were washed with PBS, added with 100 µL/pitting of MTT reagent, and incubated for 3-4 hours at 37°C. The stopper solution (10% SDS in 0.01 N HCl) was added, with a volume of 100 µL/wells. The cells were incubated overnight in the dark at room temperature and then measured with an ELISA reader at λ 595 nm to obtain absorption equivalent to the absorbance of living 4T1 cells (Rollando, 2018).

The absorbance data of the single treatment were converted into percent viability, which is a factor in the calculation of IC₅₀. Based on the IC₅₀ value, the cytotoxicity of the EEHC-EETC-cisplatin combination at various levels was tested. The results are shown in Table I.

Table I. Comparison of concentrations in the combination of *Hedyotis corymbosa* L. Extract, *Tinospora crispa* Extract, and Cisplatin

Comparison of Treatment Combinations	Single treatment	Single treatment	Single treatment
EEHC (IC ₅₀ 1/12); EETC (1/12 IC ₅₀); C (1/12 IC ₅₀)	EEHC (1/12 IC ₅₀)	EETC (1/12 IC ₅₀)	C (1/12 IC ₅₀)
EEHC (1/6 IC ₅₀); EETC (1/6 IC ₅₀); C (1/6 IC ₅₀)	EEHC (1/6 IC ₅₀);	EETC (1/6 IC ₅₀)	C (1/6 IC ₅₀)
EEHC (1/3 IC ₅₀); EETC (1/3 IC ₅₀); C (1/3 IC ₅₀)	EEHC (1/3 IC ₅₀)	EETC (1/3 IC ₅₀)	C (1/3 IC ₅₀)
KS	KS	KM	KM

Description: EEHC = the ethanolic extract of *Hedyotis corymbosa* L.; EETC = the ethanolic extract of *Tinospora crispa* roots; C = Cisplatin; KS = Control Cells; KM = Control Media

Flow cytometry

Cells (5×10^5 cells/wells) were grown in a 6-well plate, incubated for 24 hours, and treated with EEHC, EETC, cisplatin (WAKO), and their combination. For the cell cycle analysis, trypsinized adherent cells were collected and detected by adding the flow cytometry reagent (PI+RNase+Triton-X) and then incubated for 10 minutes. The apoptotic cells were detected using the FLOUS-Annexin V Apoptosis Detection Kit. The cell suspension was transferred into a flow cytometer tube, and cell cycle profiles were read by FACSCalibur flow cytometer. The Data were analyzed in the Flowing software to determine the distribution of cells in each phase of the cell cycle (Rollando, 2018).

Data Analysis

Analysis of single cytotoxic agents

Data on absorbance were converted to percent viability using the formula below:

$$\% \text{live cell} = \frac{(\text{Abs observed} - \text{Abs media control})}{(\text{Abs cell control} - \text{Abs media control})}$$

Cell viability was analyzed in Microsoft Excel 2007 to obtain linearity (r) and IC₅₀ from the curve of concentrations (log values) and the percentage of cell viability. The r -value was compared with the table ($p < 0.05$). The effectiveness of the test compound was determined by the percentage of reduction in cell viability (Rollando, 2018).

Comparative analysis of cell viabilities in the EEHC-EETC-Cisplatin combination treatment

The cytotoxic effects of the mixture were conducted by MTT assay. The combined effects were identified by calculating the Chou's Combination Index and Drug Reduction Index (DRI) (Reynolds and Maurer, 2005) for EEHC, EETC, and cisplatin in CompuSyn program.

$$CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2} + \frac{(D)3}{(Dx)3}$$

Description: (Dx) 1, (Dx) 2, and (Dx) 3 denote the concentration of single compounds for combination treatment (x). (D)1, (D)2, and (D)3 denote the concentration of each test compound used to examine the combination.

Table II. Combination index (CI) interpretation (Matthews *et al.*, 2017)

CI values	Interpretations
<0.1	Powerful synergistic effect
0.1-0.3	Strong synergistic effect
0.3-0.7	Synergistic effect
0.7-0.9	Mild-moderate synergistic effect
0.9-1.1	Nearly additive effect
1.1-1.45	Mild-moderate antagonistic effect
1.45-3.3	Antagonistic effect
> 3.3	Strong-very strong antagonistic effect

Apoptosis and cell cycle analyses

The data on flow cytometry show the percentage of cells in four quadrants, namely LL (lower left, representing the percentage of live cells), LR (lower right, the percentage of early apoptotic cells), UL (upper left, the percentage of necrotic cells), and UR (upper right, the percentage of late apoptotic cells). These data were analyzed to identify the distribution of cells in each phase of the cell cycle (i.e., G1, S, and G2/M). The inhibition of the cell cycle can be detected by comparing the therapeutic effects of the test solution on control cells.

RESULTS AND DISCUSSION

Chemical compounds of the test extracts

The chemical examination was carried out to confirm that EETC and EEHC contain alkaloids and terpenoids, which have been scientifically proven to exhibit cytotoxic activity (Lin *et al.*, 2002). The results of the identification test using Cerium (IV) Sulfate hydrate as the reagent showed that the EETC had an HRF of 5, while EEHC had HRF values of 16 and 25 (Figure 1).

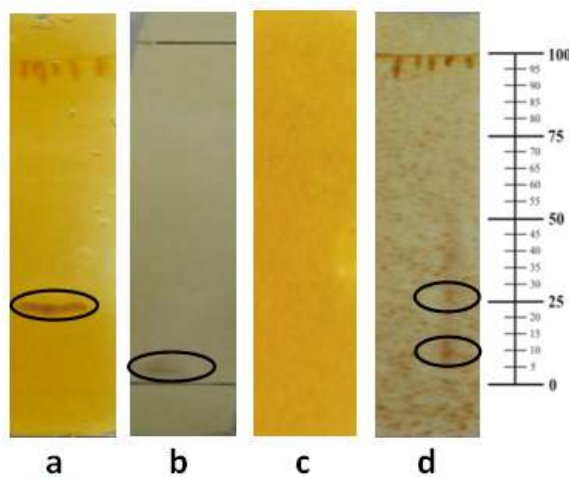


Figure 1. Test results with the Dragendorff's reagent for (a) EEHC and (c) EETC. Test results with Cerium (IV) Sulfate for (b) EETC and (d) EEHC. Elution was carried out with silica gel 60 F254 as the stationary phase and the mixture of chloroform: methanol (9: 1) as the mobile phase. The detection was carried out using visible light

The above results indicate that the plant extracts are both carbon atoms (Harborne, 1987). When the EETC plate was sprayed with the Dragendorff's reagent, a reddish-orange stain with an HRF of 25 appeared. On the contrary, there was no stain formed on the EEHC plate. In this case, orange-brown stains represent a positive reaction for alkaloids (MOH, 1985). Accordingly, EETC contains alkaloids, whereas EEHC does not. Other compounds found in both extracts are one type of triterpenoid in EETC (Aprianto *et al.*, 2017) and two triterpenoids in EEHC, namely, cycloeucalenol and cycloeucalenone (Waqas *et al.*, 2016).

Single cytotoxic activity test results of EEHC, EETC, and cisplatin against 4T1 Cells

Cytotoxic tests were designed to determine the potential of EEHC, EETC, and cisplatin in inhibiting breast cancer cells, 4T1. The parameter observed was IC_{50} , i.e., the concentration at which a substance triggers the cellular death of 50% of the population.

Table III. IC_{50} values of EEHC, EETC, and cisplatin in 4T1 Cells

Samples	IC_{50}
EEHC	5.98 ± 1.26 mg/mL
EETC	14.35 ± 0.54 μ g/mL
Cisplatin	5.04 ± 0.75 μ g/mL

Treatments with EEHC (Figure 2b), EETC (Figure 2c), and cisplatin (Figure 2d) showed a more significant decrease in the live-cell population compared to controls (Figure 2a). The cell size shrank due to the loss of cytoplasmic fluid—i.e., the result of membrane disruption, as shown by the detached cell after the above treatments (Florea and Busselberg, 2011).

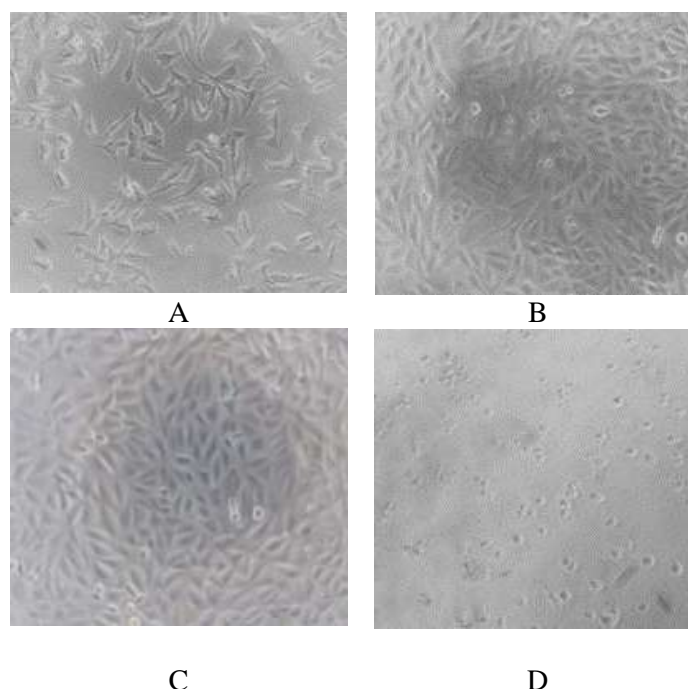


Figure 2. The Effects of Various Treatments on 4T1 Cells. A total of 8000 cells/wells in 96-well plates after 24 hours of incubation in high-glucose DMEM media. Observations were carried out under an inverted microscope with 100x magnification. (A) Control cells; (B) 5.98 μ g/mL EEHC; (C) 14.35 μ g/mL EETC, (D) 5.04 μ g/mL Cisplatin

Cytotoxic activity test results of the EEHC-EETC-cisplatin combination against 4T1 Cells

This study used a series of concentrations for each test substance, namely, 0.5, 1, and 2 mg/mL EEHC, 2.5, 5, and 10 mg/mL EETC, and 1.25, 2.5, and 5 μ L cisplatin. The combination of EEHC, EETC, and cisplatin required to produce the given effects (i.e., 1/12, 1/6, and 1/3 IC_{50}) produced CI of <1 (Table 4). In other terms, this combination has a synergistic effect.

Table IV. The combination index (CI) values of cisplatin-EETC-EEHC for 4T1 cells

Concentration ratio	Cell viability (%)	CI
1/12 IC_{50}	52.86	0.43
1/6 IC_{50}	45.76	0.58
1/3 IC_{50}	39.87	0.86

Modulation of 4T1 cell cycle in the EEHC-EETC-cisplatin combination treatment

DNA synthesis in cancer cells through the cell cycle is similar to that of healthy cells (Foster, 2008). One of the main targets in inhibiting the proliferation of cancer cells in the modulation of the cell cycle is observable by flow cytometry (King, 2000). Flow cytometry can detect each phase of the cell cycle based on the number of chromosomes in each phase (i.e., G_1 , S, and G_2/M) (Foster, 2008). Propidium iodide was used to color the cells in each phase (Ross *et al.*, 2003). The cell cycle profile was observed at the 24th hour (King, 2000). The percentage distribution of the cell cycle is presented in detail in Table V.

Table V. The Distribution of Cell Cycle (in percent)

Samples	G1 (%)	S (%)	G2 / M (%)	% CV
Control	48.98	11.62	24.68	8.87
A (1 ug/ml EEHC)	42.82	17.92	16.83	9.81
B (6 ug/ml EETC)	34.83	13.82	24.83	8.98
C (1.68 μ M Cisplatin)	43.87	11.24	29.93	9.81
The combination of A, B, and C	24.82	29.98	15.98	7.87

The test results showed that single treatments with EEHC, as well as cisplatin, caused accumulation in S phase, whereas EETC caused accumulation in S and G_2/M phases. Meanwhile, when compared to the control cells, the combination of EEHC, EETC, and cisplatin caused a collection of cells in the S phase. In the combination treatment, the percentage distribution of the S phase was 29.98% or higher than single treatments with cisplatin, which amounted to 11.24%. The combination treatment result increased on the S phase (29.98 %), and the cells accumulate in S phase due to cell cycle arrest in this phase.

Observation of apoptosis in the EEHC-EETC-cisplatin combination treatment

Induced apoptosis was used to understand the mechanism of 4T1 cell death caused by EEHC, EETC, cisplatin, and their combinations after 24 hours of incubation. Apoptosis was observed from the EEHC-EETC-Cisplatin combination at 1/6 IC_{50} concentration. Annexin V is a member of the family of phospholipid-binding protein on negatively charged cell membranes. Cell death caused by apoptosis or necrosis can be distinguished by staining propidium iodide (PI) through intercalation with DNA (Zhang *et al.*, 1997).

Table VI. The percentage of 4T1 cell mortality after treatment

	Control	EEHC (1µg/mL)	EETC (6 mg/mL)	Cisplatin (2.5 µM)	EEHC 1 ug/mL + EETC 6 mg/mL + Cis 5.3 µM
Early apoptosis (%)	1.45	2.34	1.52	2.64	10.92
Late Apoptosis (%)	1.63	0.62	2.13	2.53	1.24
Necrosis (%)	0.86	0.65	0.98	0.87	1.62
Total	3.94	3.61	4.63	6.04	13.78

After single and combination treatments (Table VI), the cell death of the control cell is at a rate of 3.94%. Single treatments using EEHC, EETC, and cisplatin caused, respectively, 3.61%, 4.63%, and 6.04% of cell death, whereas their combination resulted in 13.78% of cell death. These results prove that compared to the single use of cisplatin, the combination treatment can increase cell death by 7.4%. The extract can inhibit the phosphorylation of signal transducers and activate STAT, which is a prerequisite for the transcription process (Rabbi *et al.*, 2008).

At the molecular level, c-Myc decrease transcription, mitochondrial biogenesis, the biosynthesis of RNA and proteins, anaplerosis, and glutaminolysis and increase gene inhibitors. Meanwhile, at the cellular level, they reduce cell proliferation, transformation metabolism, and the capacity of metastasis (Miller *et al.*, 2012).

CONCLUSION

The optimum combination of the ethanolic extract of *Hedyotis corymbosa* L. (1/6 IC₅₀, 1µg/mL), the ethanolic extract of *Tinospora crispa* (1/6 IC₅₀, 6µg/mL), and cisplatin (1.68 mg/mL) can enhance the cytotoxic effects of cisplatin against breast cancer cells, chiefly 4T1. It has a synergistic effect with CI of 0.58, induces S-phase cell cycle arrest in 4T1 cells, and increases the induction of apoptosis in these cells.

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REFERENCES

- Damu, A.G., Kuo, P.C., Shi, L.S., Li, C.Y., Su, C.R., and Wu, T.S., 2009, Cytotoxic Berberina alkaloids from the stem of *Tinosporacrispa*, *Planta Medica*, 75: 1152-1156.
- Miller.M.D., Shelia D. Thomas., Ashraful Islam. David Muench and KaraSedoris., 2012, c-Myc and cancer metabolism, *Clinical Cancer Research*, 18 (20): 5546-5553.
- Florea, M.A., and Busselberg, D., 2011, Cisplatin as an anti-tumor drug: cellular mechanism of activity, drug resistance, and induced side effect. *Cancer*, 3: 1351-1371.
- Foo, S.Y., and Nolan, G.P., 1999. NF-kappaB to the Rescue: reels, Apoptosis and Cellular Transformation. *Trends in Genetics*, 6: 229-235.
- Foster, I., 2008, Cancer: A Cell Cycle Defect. *Radiography*, 14: 144-149.
- Harborne, J.B., 1987, *Phytochemical Methods: Analyzing the Modern Way Guidance Plant*. ITB Bandung, Bandung.
- Matthews, H., Jon Deakin, May Rajab, Maryam Idris-Usman, Niroshini J. Nirmalan, 2017, Investigating antimalarial drug interactions of emetine dihydrochloride hydrate using CalcuSyn-based interactivity calculations, *PLoS ONE*, 12(3): e0173303.
- IARC, 2012, Globocan 2012: Estimated Incidence of Cancer, Mortality and Prevalence Worldwide in 2012. Retrieved on August 2016.
- Jamieson, E.R, and Lippard, S.J., 1999, Structure, recognition, and processing of cisplatin DNA adducts. *Chemical Reviews*, 99(9): 2467-2498.

- King, RJB, 2000, *Cancer Biology*, 2nd ed. Pearson Education Limited, London.
- Kaur, P., Nagaraja, G.M., Zheng, H., Gizachew, D., Galukande, M., Krishnan, S., Asea, A. 2012, A mouse model for breast cancer triple negative tumor-initiating cells (TNBTICs) Similar exhibits aggressive phenotype to the human disease. *BMC Cancer*, 12:120: 1-12.
- Kemper, K., Goeje, P.L., de Peeper, D.S., Amerongen, R. van. Phenotype, 2014, Switching: tumor cell plasticity as a resistance mechanism and targets for therapy. *Cancer Research*, 74(21): 5937-5941.
- Lin, W.P., Rao, H., Su Chia Sheng, KC, Shung, and Wu, T., 2002, Phenanthroindolizidine alkaloids and their cytotoxicity from the Leaves of *Ficus septica*. *Heterocycles*, 57(12): 2401-2408.
- Milosavljevic, N., Duranton, C., Djerbi, N., Puech, P., Gounon, P., Lagadic Gossman, D., Dimanche-Boitrel, M.T., Rauch, C., Tauc, M., Counillon, L., Poet, M., 2010, Effects of Cisplatin Nongenomic: Acute Inhibition of Mechanosensitive Transporters and Channels Without actin Remodeling. *Molecular and Cellular Pathobiology*, 70(19): 7514-7522.
- Rollando, 2018, Combination *Hedyotis corymbosa* L. and *Tinospora Crispa* Ethanolic Extract Increase Cisplatin Cytotoxicity on T47d Breast Cancer Cells, *Asian Journal of Pharmaceutical and Clinical Research*, 11(7): 171-177.
- Minami, M., Matsumoto, S., and Horiuchi, H., 2010, Cardiovascular Side-effects of Modern Cancer Therapy, *Circulation Journal*, 74(9): 1779-1786.
- Rabbi, T., Anupam Bishayee, 2008, terpenoids and breast cancer chemoprevention (review). *Breast Cancer Research and Treatment*, 115: 223-239.
- Reynolds, C.P., and Maurer, B.J., 2005, Evaluating response to antineoplastic drug combinations in tissue culture models, *Methods in Molecular Medicine*, 110: 173-183.
- Ross, J.S., Linette, G.P., Stec, J., Ross, M.S., Anwar, S., dan Boguniewicz, A., 2003, DNA ploidy and cell cycle analysis in breast cancer, *American Journal of Clinical Pathology*, 120: 572-584.
- MOH, 1985, *Indonesian Medicinal Plants*, I. Ministry of Health of the Republic of Indonesia, Jakarta.
- Aprianto, Y., Asri, M., Fadliyah, H., Murwanti, R., Meiyanto, E., 2017, antigenotoxic activity of pearl grass (*Hedyotis corymbosa* L.) Ethanolic extract on cyclophosphamide-induced mice, *Indonesian Journal of Cancer Chemoprevention*, 8(3): 127-136.
- Valeriote, F., Grieshaber, CK, Media, J., Pietraszkewicz, H., Hoffmann, J., Pan, M., McLaughlin S., 2002, Discovery and Development of Anticancer Agents from Plants. *Journal of Experimental Therapeutics and Oncology*, 2(4): 228-236.
- Waqas, A., Male, I., Bukhari, N., S., 2016, *Tinospora crispa* (L.) Hook. f. & Thomson: a review of its ethnobotanical, phytochemical, and pharmacological aspects, *Front Pharmacol*, 7(59): 1-19.
- WHO, 2013, Cancer. Retrieved on August 2016.
- Zhang, G., Gurtu, V., Cain, S.R., and Yan, G., 1997, Early detection of apoptosis using a fluorescent conjugate of annexin V, *BioTechnique*, 23(3): 525-531.
- Zhao, L., Wientjes, MG, and Au, JL, 2005. Evaluation of combination chemotherapy: integration of nonlinear regression, curve shift, isobologram, and combination index analyses, *Clinical Cancer Research*, 10(3): 7994-8004.

