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Combination of *Solanum nigrum* L. Herb Ethanolic Extract and Doxorubicin Performs Synergism on T47D Breast Cancer Cells

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Abstract

Leunca (*Solanum nigrum* L.) has been proven to possess anticancer activity on some type of cancer cells. In vitro study of solamargine found in the herb showed cytotoxic effect against several breast cancer cell lines, such as T47D and MDA-MB-31. Hence, further study on its potential as a co-chemotherapeutic agent needs to be conducted, in order to overcome resistance problem commonly found in cancer chemotherapy. This study aimed to examine the cytotoxic activity of leunca herb ethanolic extract (LEE) alone and its combination with doxorubicin. Single and combinational treatment of LEE and doxorubicin on T47D breast cancer cells were done, and their viability representing cytotoxicity were analyzed by using MTT assay to determine the IC_{50} value and combination index (CI) to evaluate the combinational effect. Twenty four hours-treatment of LEE alone gave cytotoxicity activity showing a dose-dependent manner with the IC_{50} of 47 µg/ml, while combinational treatment showed that 4 µg/ml LEE was found to be synergist with 4 nM doxorubicin on T47D cells, with the optimum CI value of 0.59. This result shows that *Solanum nigrum* L. is potential to be proposed as doxorubicin co-chemotherapeutic agent against breast cancer. Further study on its molecular mechanism needs to be conducted.

Key words: *Solanum nigrum*, doxorubicin, synergist, breast cancer

INTRODUCTION

Doxorubicin, a chemotherapy agent that is applicable for the treatment of several cancer types, is widely used in the treatment of breast cancer. Medical report by Rumah Sakit Kanker Dharmais stated that in 2007 breast cancer shows the highest incidence compared to another cancer types (Anonym, 2009). The main problems being faced in the application of chemotherapeutic agent are its toxicity towards normal tissues, suppression of the immune system (Wattanapitayakul *et al*., 2005), and occurrence of resistance (Mechetner *et al*., 1998). One promising approach to solve this problem is the application of co-chemotherapeutic agent in cancer therapy.

Co-chemotherapy may increase chemotherapeutic agents' efficacy, allowing the use of lower dosage of chemotherapeutic agent, resulting in the decrease of toxicity on normal tissues. Potent agent which can be use as co-chemotherapeutic agent together with doxorubicin are phytochemical substances, rendering to the fact that there are huge number of substances found in plants that are potential against cancer (Gibbs, 2000), such as solanine, solasodine, and solamargine found in leunca (*Solanum nigrum* L.) herb (Everist, 1974; Weller and Phipps, 1979). Those steroidal glycosides have been proven to possess anticancer activity (Saijo *et al*., 1982). Solamargine as the main constituent being responsible for leunca's anticancer activity (Hu, 1999) performed cytotoxic activity on several cancer cell lines, including T47D and MDA-MB-231 breast cancer cells. Besides, it also induces programmed cell death, apoptosis, through mitochondrial pathway (Liang, 2008). Therefore, further study on its potential as co-chemotherapeutic agent needs to be conducted.

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This study aimed to observe the effect of leunca's ethanolic extract (LEE) application to the cytotoxicity performed by doxorubicin, analyzed by using MTT assay. Combinatorial treatment of doxorubicin and LEE were applied in order to increase the cytotoxicity of doxorubicin on T47D cells, allowing the use of lower dose of the chemotherapeutic agent giving less toxicity on normal tissues.

METHODS

Sample preparation

Dried powder of leunca herbs were purchased from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TOOT), Indonesia. Dried powder was then extracted by maceration for 5 days with 70% ethanol. Collected filtrate was concentrated using rotary evaporator (Heidolph WB2000), then dissolved in Dimethyl Sulfoxide (DMSO) (Sigma). Both Doxorubicin 5mg/ml and extract solution were diluted in DMEM cell culture medium before being applied.

Chemicals

Dulbecco's Modified Eagle Medium (DMEM) powder (Gibco), Fetal Bovine Serum (FBS) 10%$^{v}/_{v}$ (Gibco), and Penicillin-Streptomycin 10,000 units/ml penicillin-10,000 µg/ml streptomycin (Gibco) were used for cell culture medium. Cells were prepared using Tripsin EDTA 25% (Gibco). For cytotoxicity assay, Sodium Dodecyl Sulphate (SDS) 10%$^{v}/_{v}$ (Merck) dissolved in HCl 0.1N (Merck) as stopper reagent, Phosphate Buffer Saline (PBS) pH 7.4 containing KCl (HPLC grade, Sigma), NaCl (HPLC grade, Sigma), Na_2HPO_4 (HPLC grade), and KH_2PO_4 (HPLC grade, Sigma) dissolved in aquadest as washing reagent, and 3-[4,5-dimethyl thiazole-2-yl(-2,5- diphenyltetrazoliumbromide)] (MTT) dissolved in PBS as MTT reagent were used.

T47D cells

T47D cells being used were from the collection of Cancer Chemoprevention Research Center (CCRC), Universitas Gadjah Mada. The cell line was a gift from Prof. Kawaichi, *Nara Institute of Science and Technology* (NAIST), Japan.

Cytotoxicity and combinational assay

MTT cytotoxicity assay was used to examine the effect of LEE alone and in combination with doxorubicin on T47D cells. T47D cells was distributed to 96-well plate with the density of $5x10^3$ cells/well and incubated in 37˚C with 5% CO_2 for 24 hours. For the cytotoxicity assay of LEE on T47D cells alone, LEE was applied with the concentration of 1, 10, 50, 100, 250, 500, and 750 µg/ml to find IC_{50}. In combinational assay, concentration of about $\frac{1}{2}, \frac{3}{8}, \frac{1}{4}$ and $\frac{1}{8}$ IC_{50} acquired were used for both LEE and doxorubicin, giving the concentration of 18, 12, 6, and 4 µg/ml for LEE and 6, 4, 2, and 1.5 nM for doxorubicin. After 24 hours incubation, MTT reagent was applied, followed by 4 hours incubation. SDS 10%$^{v}/_{v}$ in HCl 0.1N as stopper reagent was then applied. Plate was then kept with protection from light overnight, continued with absorbance determination (λ 595 nm) using ELISA reader (Bio-Rad).

Analysis

Single Cytotoxicity assay

Linear regression between log concentration and % cell viability giving the equation y = Bx + A were used to calculate IC_{50} value, that is the concentration inhibiting 50% cell proliferation.

Combinational Cytotoxicity Assay

Combinational treatment was evaluated by calculating Combination Index (CI) value (Reynolds and Maurer, 2005), which has the formula as follows.

$$CI = \frac{D1}{Dx1} + \frac{D2}{Dx2}$$

D1 and D2 represent concentrations used in combinational treatment, while Dx1 and Dx2 are single treatment concentration giving the same response as D1 and D2, respectively. CI value acquired will allow the evaluation of LEE's potency in combinatorial treatment with doxorubicin on T47D cell lines. Interpretation was done based on the classification listed in table 1.

Table 1. Interpretation of CI value representing potency of combinational application

CI value	Interpretation	CI	Interpretation
< 0.1	Very strongly synergist	0.9-1.1	Closely additive
0.1-0.3	Strongly synergist	1.1-1.45	Middle antagonist
0.3-0.7	Synergist	1.45-3.3	Antagonist
0.7-0.9	Middle synergist	> 3.3	Strongly antagonist

RESULTS

Treatment of LEE on T47D cells gave the value of cell viability shown in table 2. Increasing LEE concentration resulting in the increase of cytotoxicity. Linear regression of LEE concentration against % cell viability (figure 1) gave the IC_{50} value of 47 µg/ml. Doxorubicin's IC_{50} on T47D cells is 15 nM (Junedi, 2010). Observation of cell morphology after treatment was also done, shown in figure 2. Increasing concentration of LEE was followed by the increasing number of dead cells, quantitatively. Both qualitative and quantitative data prove that single treatment of LEE on T47D cells showed a dose-dependent manner.

Combinational treatment of LEE and doxorubicin on T47D cells gave the CI value shown in table 3, and being plotted in diagram (figure 3). It shows that 4 concentration combinations, that are doxorubicin $^1/_8IC_{50}$ – LEE ½ IC_{50}, doxorubicin $^3/_8IC_{50}$ – LEE $^1/_8IC_{50}$, doxorubicin $^3/_8IC_{50}$ – LEE ¼ IC_{50}, and doxorubicin $^3/_8IC_{50}$ – LEE ½ IC_{50}, combination of shows synergicity on T47D cells, with CI values less than 0.9. Cell morphology after treatment was observed (figure 4). Treatment of LEE and doxorubicin alone led to cells' morphological change pointed by black arrows, respectively (figure 4(b) and 4(c)). Combination of them caused more changes compared to single treated cells (figure 4(d)). While control cells showed no change in cells' morphology representing cells' death (figure 4(a)). Hence, synergism of combinational treatment was observed.

Table II. % cells viability in single treatment of LEE on T47D cells.

LEE concentration (µg/ml)	% cell viability
750	2.33
500	4.53
250	9.91
100	43.94
50	73.81
10	76.01
1	102.57

DISCUSSION

This study had observed that treatment of LEE on T47D breast cancer cells could inhibit cells' growth with a dose-dependent manner. Higher dose of LEE brought less cell viability, showing higher toxicity. LEE IC_{50} value on T47D cells of 47 µg/ml was acquired. It represents LEE's efficacy as a single cytotoxic agent. According to Ueda, 2000, IC_{50} value less than 100 µg/ml shows that related agent has a potent cytotoxicity. The mechanism of cytotoxic activity of solanine and solamargine, two steroidal glycosides found in leunca herb, had been previously studied. However, its specific molecular target has not been discovered yet.

Previous study proved that solamargine and solanine could suppress Bcl-2 protein expression (Liu et al., 2004; Ji et al., 2008). Bcl-2 functioned as antiapoptotic protein that inhibit cells' death through apoptosis, thus its suppression may trigger cancer cells' death (Pandanilam, 2003). Bcl-2 suppression plays a role in apopotosis induction by triggering mitochondrial release of cytochrome c which later activates the caspase

cascade. Hence, one possible mechanism of LEE's cytotoxic activity is by apoptosis induction. Apoptosis may occur involving the role of several types of proapoptotic proteins, such as Bad, Bax, and Bak (Ricci and Zong, 2006). Further study needs to be conducted to observe whether or not LEE triggers apoptosis and also its ability to affect the expression of both proapoptotic and antiapoptotic proteins other than Bcl-2 on T47D cells.

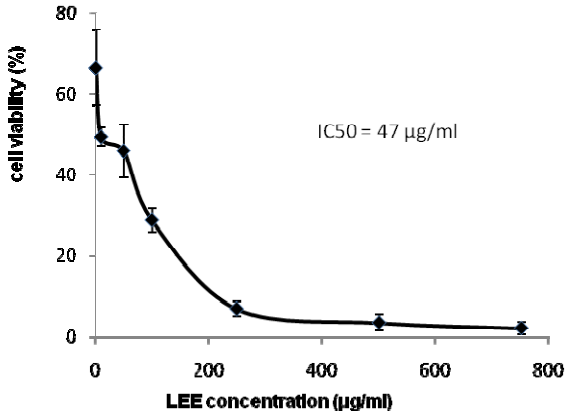

Figure 1. Curve of log LEE concentration against % cell viability on T47D cells, showing a dose-dependent manner. Linear regression gave the equation y = -37.56x 112.71, giving IC_{50} value of 47 µg/ml.

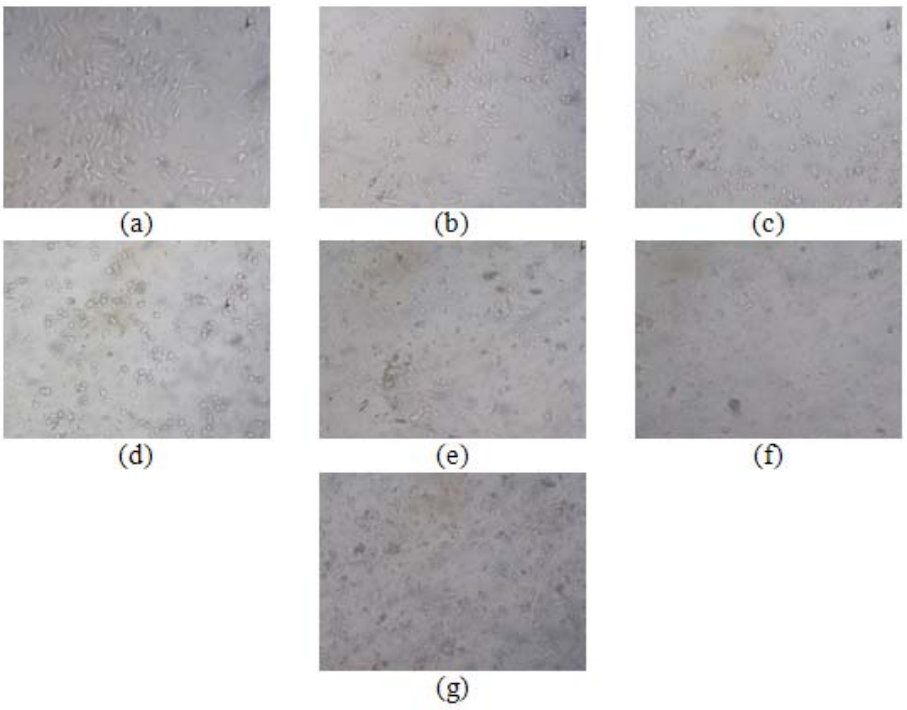

Figure 2. T47D cells' morphology after 24 hours LEE treatment;(a) 1 µg/ml; (b) 10 µg/ml; (c) 50 µg/ml; (d) 100 µg/ml; (e) 250µg/ml;(f) 500 µg/ml; (g) 750 µg/ml.Higher concentration of LEE caused more change in cells' morphology (round cells being unattached to the well). This phenomenon represents a dose-dependent manner.

Table III. CI values of combinational treatment of LEE and doxorubicin on T47D cells.

LEE concentration (µg/ml)	Doxorubicin (nM)			
	1.5	2	4	6
4	1.54	1.41	**0.59**	1.83
6	2.92	2.04	**0.81**	2.43
12	0.97	2.05	1.27	1.86
18	**0.79**	1.14	**0.75**	1.31

Bold numbers shows combination resulting in CI value below 0.9, which are synergist.

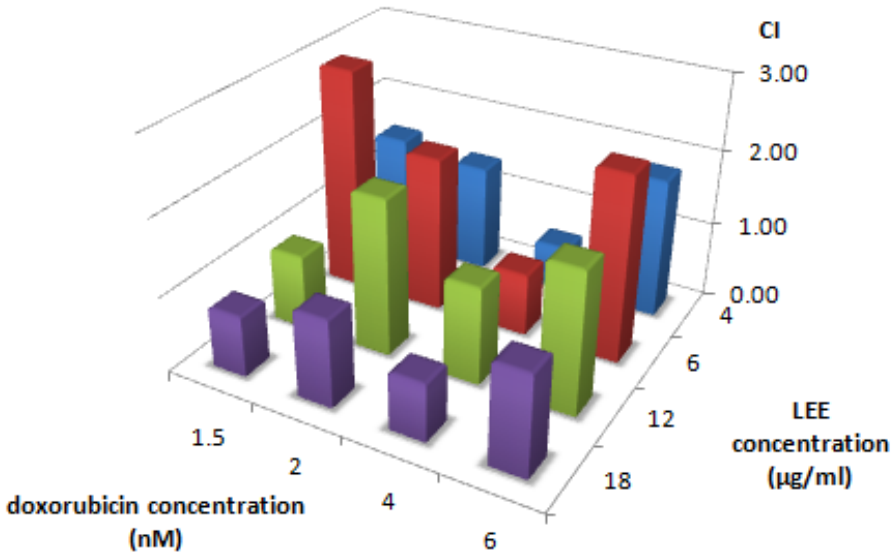

Figure 3. Diagram of combinational effect of LEE and doxorubicin against CI value. CI value below 0.9 shows sinergicity.

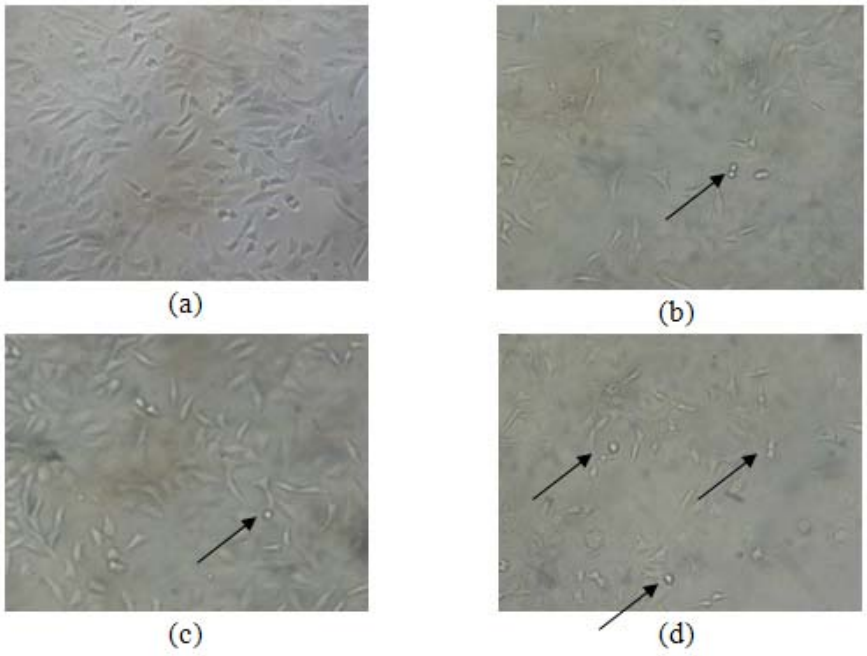

Figure 4. T47D cells' morphology after 24 hours treatment; (a) cell control; (b) LEE 4µg/ml; (c) doxorubicin 4 nM; (d) doxorubicin 4 nM in combination with LEE 4µg/ml. Treatment of LEE and doxorubicin alone brought cells to death, shown by change in cells' morphology pointed by black arrows, respectively (figure (b) and (c)). Combination of them caused more cells' death compared to single treated cells (figure (d)), showing sinergicity. While control cells showed no change in cells' morphology representing cells' death (figure (a)).

The efficacy of combination between LEE and doxorubicin on T47D cells were analyzed to determine their combinational effect, whether they are synergist, additive, or antagonistic. It allows us to know LEE's ability to increase chemotherapeutics agents' effectiveness that is commonly used in breast cancer therapy. Result shows that four combinational doses of doxorubicin and LEE performs synergism on their cytotoxicity towards T47D cells, meaning that doxorubicin's theraputical dose may be lessen by the application of LEE as a cochemotherapeutic agent, giving equal effectiveness in inhibiting cancer cells' growth.

Synergism observed possibly occur because of the ability of LEE to strengthen doxorubicin's cytotoxic effect. In this case, doxorubicin is cytotoxic towards cells by inhibiting topoisomerase II activity (Potter et al., 2002), and was strengthened with the cytotoxic activity of solanine and solamargine, possibly by triggering apoptosis or by inducing cell cycle arrest. Another study is required to know the exact mechanism of synergism between doxorubicin and LEE. Further study on LEE apoptosis induction and its effect on related proteins expression need to be conducted in order to know more about its cytotoxic molecular mechanism.

CONCLUSION

From the study, it can be concluded that *Solanum nigrum* herbs' ethanolic extract is cytotoxic and is able to increase doxorubicin's cytotoxicity on T47D breast cancer cells.

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