

Synergistic Effect of Cinnamon Essential Oil (*Cinnamomum burmannii*) and Doxorubicin on T47D Cells Correlated with Apoptosis Induction

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Abstract

Cinnamon (*Cinnamomum burmannii*) shows anticancer activity in several types of cancer cells. The aim of this study is to observe the cytotoxic activity of cinnamon essential oil (CEO) solely and its combination with doxorubicin, also the ability of the combination to induce apoptosis on T47D breast cancer cells. The CEO was prepared through distillation of dry cinnamon bark. Cytotoxic assay was performed by using MTT assay and apoptosis determination was done by using double staining with ethidium bromide and acridine orange. The result showed that CEO exhibited cytotoxic effect on T47D cells with IC₅₀ values of 75 µg/mL. Moreover, CEO showed synergist effect with doxorubicin. The lowest combination index (CI) with CI values of 0,37 was obtained by combination of doxorubicin-CEO 37.5 µg/mL-1.25 µM. Treatment with CEO solely and its combination with doxorubicin showed apoptosis induction on T47D cells. The results of this study indicate the potency of CEO to be developed as co-chemotherapeutic agent of doxorubicin on breast cancer.

Keywords: cinnamon essential oil, doxorubicin, T47D cells, combination cytotoxic

INTRODUCTION

Cancer is one of the major human diseases and causes considerable suffering and economic loss worldwide. Breast cancers show high incidence over the world and there are estimated over 200,000 new cases of breast cancer on American women (American Cancer Society, 2013). Selective eradication of cancer cells is an important goal for cancer chemotherapy in the 21st century. Chemotherapeutic agent, such as doxorubicin, causes several side effect such as depression of immune system (Wattanapitayakul, *et al.*, 2005) and resistance of cancer cells (Smith, *et al.*, 2006).

Combination of chemotherapy (co-chemotherapy) is a strategy to make cancer therapy become more effective, safe, and suppress side effect of doxorubicin. Phytochemicals are potential to be developed as anticancer agent (Tyagi, *et al.*, 2004) and several compounds could be found in cinnamon bark. The active component of cinnamon bark, cinnamaldehyde, performs antiproliferative

activity in several cancer cells such as lung, renal, colon, CNS, melanoma, breast, leukemia, ovary, and prostate (Lee, *et al.*, 1999). The aim of this study is to observe the synergistic effect of cinnamon essential oil (CEO) combined with doxorubicin in cytotoxic effect and apoptosis induction on T47D breast cancer cells. The results of this study can be a reference for further research of developing CEO as potential co-chemotherapeutic agent.

MATERIALS AND METHODS

Cinnamon Essential Oil (CEO)

Cinnamon bark was obtained from Boyolali, Central Java, Indonesia and determined in Pharmaceutical Biology Laboratory, Faculty of Pharmacy, UGM. The barks was dried and chopped, then placed in the sample flask.

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Aquadest was added to the water flask (± 3 L) and boiled until 100°C. Steam was produced under the sample and condensed as distillate.

Cell Culture

T47D cells was obtained from Prof. Tatsuo Takeya (Nara Institute of Science and Technology, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% v/v 10,000 units/mL penicillin-10,000 µg/ml streptomycin (Gibco) at temperature 37°C and with a flow of 5% CO₂.

Cytotoxic Assay

T47D cells were seeded in 96-well plate with 1×10^4 cells/well and divided into control and treatment group. Final concentrations of CEO were 50, 100, 150, 200, 300, 400 and 500 µg/mL while concentration of doxorubicin were 5, 10, 20, 30, 40, and 50 µM. Concentration series of CEO and doxorubicin then diluted in culture medium up to the final concentrations. After 24 h incubation, culture medium was removed and cells were washed using PBS (Sigma). Five mg/mL of MTT on PBS (Sigma) was diluted by DMEM (1:9) and 100 µL of it was added into each well. After incubation for 3 hours, the reaction was stopped by Sodium Dodecyl Sulfate (SDS) 10% in HCL 0.01 N. The plate then incubated for one night in room condition at dark place. To make sure the formazan dissolve, the plate was shaken for 10 minutes and the absorbance was measured using ELISA reader at wave length of 595 nm.

Apoptosis Induction

Cover slips were placed on 24 wells tissue culture plate. T47D cells were seeded at 5×10^4 cells/well. After 24 hours of incubation, cells were treated with CEO, doxorubicin, and combination of CEO and doxorubicin. After 24 h of these treatments, cells were washed. Cover slips were taken and put on the object glass and added by 10 µL 1X *working solution* ethidium bromide-acridin orange (Kasibhatla, *et al.*, 2006). The treated cells then observed under fluorescent microscope.

Data Analysis

Single Cytotoxicity Assay. Linear regression between concentration and % cell viability giving the equation $y = Bx + A$ were used to calculate IC₅₀ 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.

$$CI = D1/Dx1 + D2/Dx2$$

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 CEO's potency in combinatorial treatment with doxorubicin on T47D cells. Interpretation was done based on classification listed in Table 1.

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

CI	Interpretation	CI	Interpretation
< 0.1	Very strong 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 AND DISCUSSION

Effect of Cinnamon Essential Oil (CEO) and Doxorubicin on T47D Cells Growth

CEO with dose range of 50-400 $\mu\text{g/mL}$ inhibited cell viability of T47D with IC_{50} value of 75 $\mu\text{g/mL}$. Along with the increase of CEO dose, cell viability was decrease (Fig.1A). CEO caused morphological changes in the cells (Fig.1C-E) while there were no morphological changes in control cells (Fig.1B).

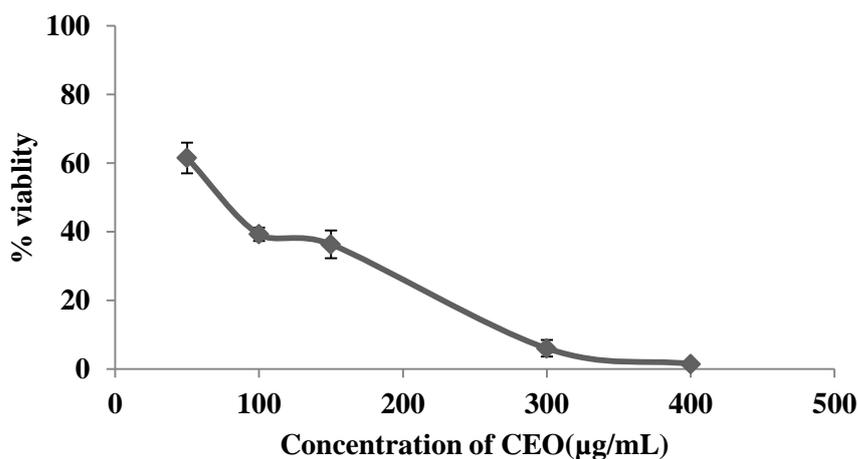
Effect of Cinnamon Essential Oil (CEO) Combined with Doxorubicin on T47D Cells Growth

Combination assay was done by combining 1/2, 1/4, 1/8, 1/20 IC_{50} of CEO and doxorubicin. The IC_{50} of CEO was 75 $\mu\text{g/mL}$ and IC_{50} of doxorubicin from the previous research was 10 μM (*unpublished data*). Thus, combination dose for CEO were 37.5; 18.75;

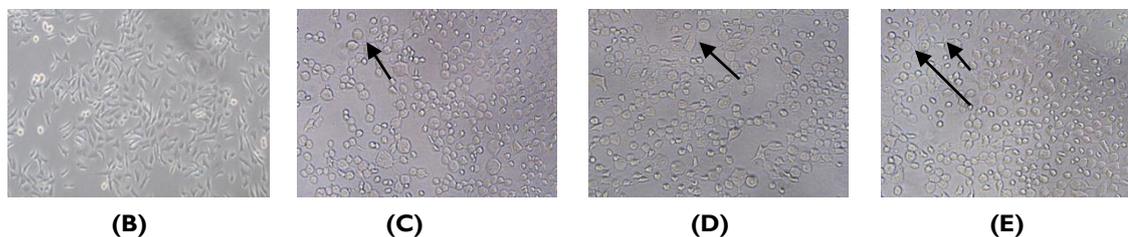
9.375; 3.75 $\mu\text{g/mL}$ and doxorubicin were 5; 2.5; 1.25; dan 0.5 μM . Combination of CEO and doxorubicin decreased cell viability compared to doxorubicin alone. Combination index (CI) was calculated to evaluate the synergistic effect of the combination.

Results showed nine combinations performed synergistic effects, with the CI values of less than 0.9 (Reynolds and Maurer, 2005). The smallest CI showed by combination of 37.5 $\mu\text{g/mL}$ CEO and 1.25 μM doxorubicin (Table 2).

Treatment with combination of CEO 37.5 $\mu\text{g/mL}$ and doxorubicin 1.25 μM reduced cell viability significantly different with single treatment of each agent. While combination treatment with lower concentration of CEO (9.375 $\mu\text{g/mL}$) and doxorubicin did not reduce cell viability significantly compound to the doxorubicin solely (Fig. 2).



(A)



(B)

(C)

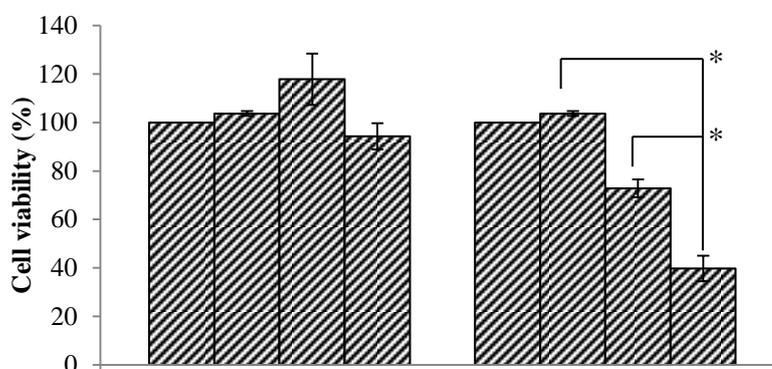
(D)

(E)

Figure 1. Effect of cinnamon essential oil (CEO) and doxorubicin on T47D cells growth. (A) Graphic between CEO dose and T47D cells viability. CEO cytotoxicity expressed by percent cell viability is shown as the mean + SE of 3 experiments. IC_{50} values obtained from the calculation of linear regression of concentration vs % viable cells with $p < 0.05$. CEO showed cytotoxicity effect on T47D cells in dose dependent manner; (B) Morphology of cells without treatment; (C) treatment of CEO 50 $\mu\text{g/mL}$; (D) treatment of CEO 150 $\mu\text{g/mL}$; (E) treatment of CEO 300 $\mu\text{g/mL}$. Arrows (\rightarrow) indicates cells undergoing morphological changes.

Table 2. Combination Index combination of CEO and doxorubicin

CEO (µg/mL)	Doxorubicin (µM)			
	0.5	1.25	2.5	5
3.75	0.52	0.64	0.57	0.50
9.375	1.59	0.68	0.60	2.68
18.75	2.81	1.56	1.32	1.17
37.5	4.90	0.37	0.42	0.53



Dox 1.25 µM	-	+	-	+	-	+	-	+
DKM 9.375 µg/mL	-	-	+	+	-	-	-	-
DKM 37.5 µg/mL	-	-	-	-	-	-	+	+

Figure 2. Effect of CEO in combination with doxorubicin on T47D cell viability. Cytotoxic assay was done under MTT reaction as described in the methods. Graph represented from the means of 3 independent experiments and analyzed with ANOVA one way with $p < 0.05$.

Effect of CEO and Doxorubicin on Apoptosis Induction

Double staining using ethidium bromide and acridin orange was used to observe the induction of apoptosis. Single treatment of CEO 37.5 µg/mL and doxorubicin 5 µM induced apoptosis of T47D cells. Apoptotic induction was indicated by orange fluorescent

in the nucleus (Fig.3, red arrows). The result showed that combination treatment of CEO and doxorubicin increased the number of cells containing red/orange fluorescent. These data indicated that the combination of CEO and doxorubicin increased the incidence of apoptosis.

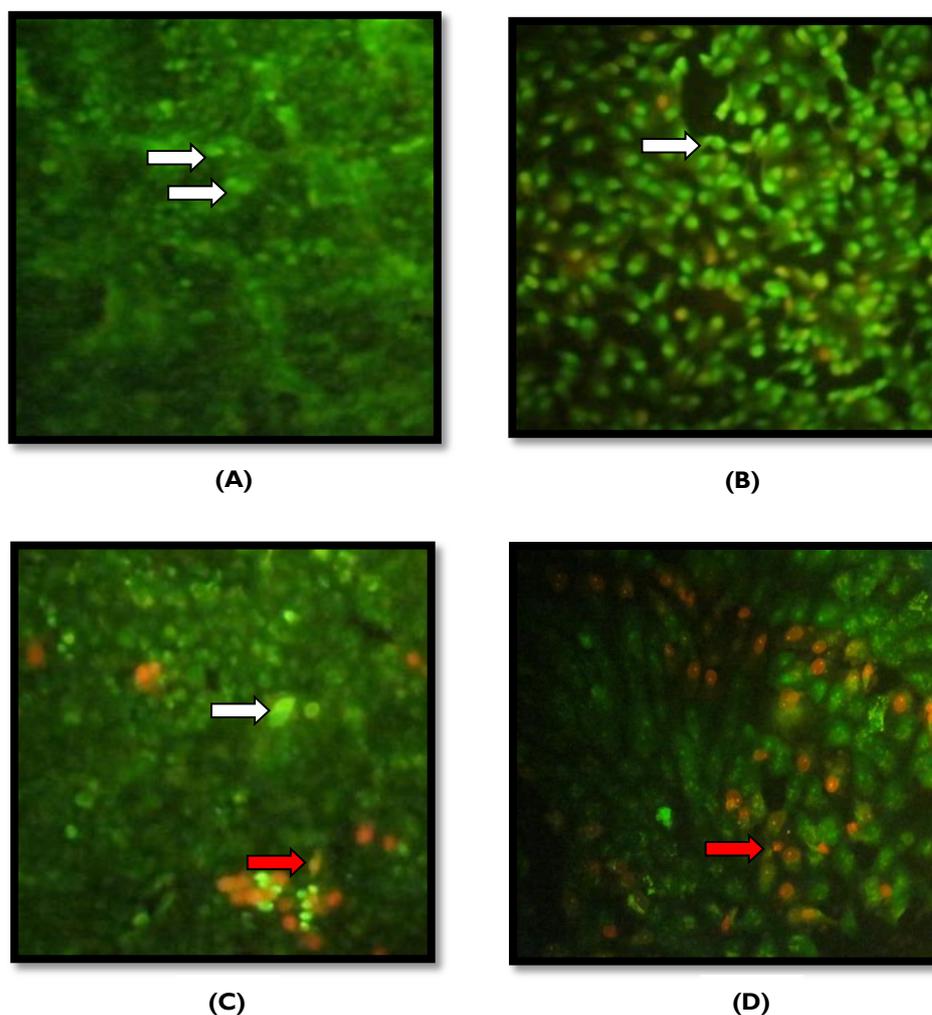


Figure 3.Effect of CEO and doxorubicin on apoptosis. (A) Morphology of cells without treatment; (B) treatment of doxorubicin 5 μ M; (C) treatment of CEO 37.5 μ g/mL; (D) treatment combination of CEO 37.5 μ g/mL and doxorubicin 5 μ M. Arrows (\blacktriangleright) viable cells that has green fluorescent, arrows (\blacktriangleright) apoptosis cells that has orange fluorescent.

CEO showed cytotoxic effect on breast cancer T47D cells. CEO inhibited T47D cells growth with IC₅₀ value of 75 μ g/mL. According to Ueda, *et al.* (2000), extract with IC₅₀ below 100 μ g/mL is potential to be developed as anticancer agent. CEO also increased cytotoxic activity of doxorubicin on T47D cells, indicated by cell viability decrease compared to doxorubicin treatment solely. This mean that when combined with CEO, doxorubicin dose could be decreased but gave the same effect on cancer cells.

Synergistic effect of combination of CEO and doxorubicin could be done through

several mechanisms, such as apoptosis induction and/or cell cycle modulation. In this study, double staining method was done to confirmed apoptosis induction by CEO and doxorubicin. From this study, combination of CEO (37.5 μ g/mL) and doxorubicin (5 μ M) increased apoptosis incidence compared to each compound solely.

Doxorubicin usually need p53 to induce apoptosis (Drummond, 2007), while T47D has mutant p53. Thus, apoptosis induction on T47D is probably through p53-independent pathway. Doxorubicin also has been known to activate NF κ B. NF κ B activation leading to expression

of anti-apoptotic protein, such as Bcl-xL (Pahl, 1999). On the other hand, aqueous extract of cinnamon has been known to inhibit NF κ B activation (Kwon, *et al.*, 2010). By NF κ B inhibition, expression of anti-apoptotic protein could be inhibited. Nevertheless, further research has to be done to observe effect of CEO and doxorubicin on expression of protein related with apoptotic pathway.

Previous study showed that cinnamaldehyde, major compound in cinnamon, induced apoptosis through mitochondrial permeability transition (MPT) (Ka, *et al.*, 2003). Increase of mitochondrial membran permeability caused cytochrome c excess, then activate caspase 3 and leading to apoptosis (Mizutani, *et al.*, 2005).

But, cytotoxic activity in CEO seems likely not only caused by cinnamaldehyde. Singh, *et al.* (2007) reported that aqueous cinnamon extract (ACE) showed stronger cytotoxic effect compared to cinnamaldehyde alone. There are some polyphenol compounds that might has synergistic effect with cinnamaldehyde. Thus, distillate standardization is needed to be done as well as to know more active compound in the CEO.

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