SYNERGISTIC COMBINATION OF *Curcuma xanthorrhiza*, *Ficus septica* AND DOXORUBICIN INHIBITS METASTASIS OF BREAST CANCER THROUGH INHIBITION MMP-9 ACTIVITY

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INTRODUCTION

Breast cancer is one of the most common malignancy on woman in Indonesia (Kemenkes RI, 2015). Breast cancer patients usually come to doctor in a late stage & the deaths are due to the complications of metastasis in vital organs (National Cancer Institute, 2012). The death case most probably happened when cancer is aggresive & distant metastazed. One factor that causing aggresivity & metastasis is presence of HER2 protein. HER2 is found in 25% cases of Ca mammae. Her2 status also influences preference of drug for cancer treatment. Besides of antibodi monoklonal & antireseptor of tirosine kinase, doxorubicin is still used for treatment breast cancer with overexpress HER2 (Nielsen et al., 2009). Long term use of doxorubicin causes several side effects, resistance and toxicity to normal tissues (Smith et al., 2010).

Therefore, combining doxorubicin with chemopreventive agent is needed to increase activity of doxorubicin, to overcome the drug resistance and to reduce its side effect (Sarkar and Li, 2006). Chemopreventive agent used in this research are temulawak (*Curcuma xanthorrhiza*) and awar-awar (*Ficus septica*). The leaves of *Ficus septica* have been used to treat colds, fungal and bacterial diseases, and various cancers. The ethanolic extract of *Ficus septica* showed a cytotoxic effect on breast cancer T47D cell lines with IC_{50} value of 13 µg/mL. The extract at 4.88 µg/mL also showed an optimum synergistic effect in combination with doxorubicin (3.75 nmol) (Pratama et al., 2010). In addition, the extract induced apoptosis and down regulated the expression of Bcl-2 protein in breast cancer cells MCF-7 (Sekti et al., 2010). The major components of *C. xanthorrhiza* are curcumin and xanthorrhizol. Curcumin suppresses a number of key elements in cellular signal transduction pathways pertinent to growth, differentiation, and malignant transformation (Kunumakkara et al*.,* 2008). Choi et al*.* (2005) observed that injection of 0.2-1.0 mg/kg bw xanthorrhizol had an antimetastatic effect in a mouse lung metastasis model.

It is a general assumption that combination treatment is sometimes more beneficial than single compounds. The previous study reported that combination of doxorubicin and chemopreventive agent showed synergistic effect in cancer treatment (Lewandowska et al., 2014). The purpose of this study is to prove antimetastasis effect of combination of ethanolic extract of rhizome of *Curcuma xanthorrhiza* (ECx), ethanolic extract of leave of *Ficus septica* (EFs), and doxorubicin solely and its combination on MCF7-HER2 breast cancer cell line through inhibition activity of MMP-9. Cytotoxic assay is used to determine the synergy of combinations. Antimetastasis effect was observed through inhibition of cancer cell invasion. Gelatin zymography signify the inhibitory activity of MMP-9, which plays an important role in cancer cell invasion.

METHODS

Cells culture

MCF7 cells transfected HER2 gen and 4T1 murine mammary carcinoma cells were acquired from Prof. Masashi Kawaichi (Nara Institute of Science and Technology, Japan) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose containing Fetal Bovine Serum (FBS) 10% (v/v) (Sigma), penisilin-streptomisin 1% (v/v) (Gibco) and Fungizone 0.5% v/v (Gibco) in a humidified atmosphere of 5% CO_2 in air 37 °C.

Cytotoxicity assay

Cytotoxic effect of ECx, EFs and doxorubicin in a single and combination on MCF7-HER2 breast cancer cells was evaluated by MTT colorimetric assay. 12 thousand mcf7her2 cells/well was distributed into 96 well plate and incubate for 24h. After 24 hours treatment of various concentrations of samples (ECx, EBj, EFs, Dox and its combination), cells were given 0.5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Biovision) and incubated further for 4 hours. Cells were lysed using SDS stopper containing 0.01 N HCl and incubated in the dark condition for overnight. Percent cells viability was determined by converting the

absorbance data of the cell treatment on wavelength 595 nm. It was used to determine the IC_{50} value. Afterwards, cytotoxicity assay was also conducted to determine the effect of combination of various sample concentrations treatment (Mosmann, 1983). The effect of combination treatment on MCF7-HER2 cells was evaluated by calculating Combination Index (CI) by using CompuSyn software.

Gelatin zymography

Antimetastasis effect was observed through inhibition of cancer cell invasion. Gelatin zymography signify the inhibitory activity of MMP-9, which plays an important role in cancer cell invasion. The SDS-PAGE 8% supplemented with 0.1% gelatin was used to determine the activity of MMP-9 in the culture medium. After electrophoresis running, gels were washed and incubated with distilled water containing 2% Triton-X 100 (Merck) for 30 minutes in room temperature. The solution was removed from gels and 100 mL of reaction buffer (40 mM Tris-HCl pH 8, 10 mM CaCl2, 0.02% NaN_3) was added, and then it was incubated for 24 hours at 37°C. After the removal of the reaction buffer, gels were stained by using Coomassie Brilliant Blue R-250 (Sigma) and destained using destaining solution (20% methanol, 10% acetic acid and 70% water) until clear bands with dark blue background appeared (Kupai, 2011). The results were documented and analyzed by using Image J software.

Statistical analysis

Oneway ANOVA followed by the least significant difference (LSD) were used to assess the statistical significance of difference between untreated and different treatment group. A statistically significant difference was considered to be present at $p< 0.05$. The results of scratch wound healing assay and gelatine zymograph were documented and analyzed by using Image J software before ANOVA.

Combination cytotoxicity assay and synergicity

The effect of combination treatment on 4T1 cells was evaluated by calculating Combination Index (CI) value (Reynolds and Maurer, 2005) using the formula as follows:

$$C1 = \frac{D1}{Dx1} + \frac{D2}{Dx2} + \frac{D3}{Dx3}$$

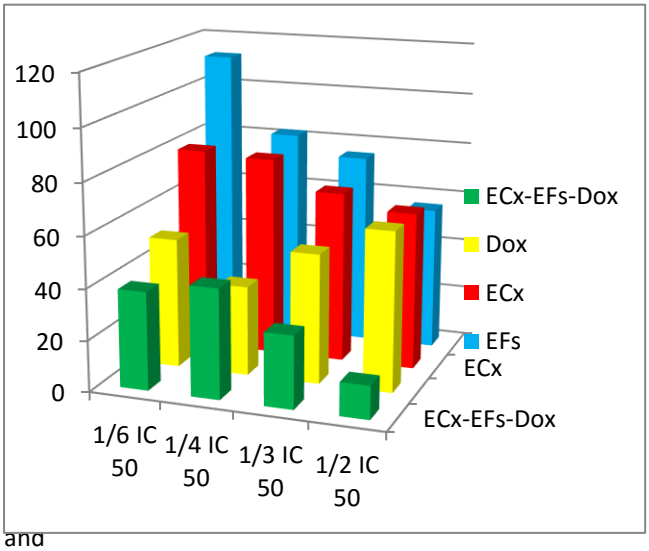
D1, D2, and D3 represented the concentrations used in combinationtreatment, while Dx1, Dx2, and Dx3 are the single treatment concentration giving the same response as D1, D2 and D3 respectively.

RESULTS

Exposure of ECx, EFs, Dox and its combination at various concentrations for 24h showed cell proliferation inhibition in a concentration-dependent manner. A single treatment of ECx, EFs, or Dox showed cytotoxic effect on MCF7-HER2 breast cancer cells with IC_{50} value respectively 180 µg/mL, 48 µg/mL, and 4.8 µg/mL (Figure 1). The reduction of cell viability of combination of ECx, EFs and Dox is greater than the single treatment. The combination of ECx-EFs-Dox using concentrations of ½ IC_{50} (90 µg/mL of ECx, 24 µg/mL of EFs, and 2.4 µM Dox showed synergistic effects with CI value < 1 (Table 1) and reduced cell viability up to 57% (Figure 2).

Table 1. CI values of various concentration of ECx,EFs and doxorubicin

No.	Dose	Viability	CI Value
1.	$^1/_6$ IC 50	38,31	0.19
2.	$^1/_4$ IC 50	42,72	0.35
3.	$^1/_3$ IC 50	28,21	0.29
4.	$^1/_2$ IC 50	12,91	0.30



and

Figure 2. Viability of MCF7-HER2 cells due to single and combination treatment of ECx,EFs and doxorubicin. $12x10^3$ cells per well were incubated for 24h and exposed with various concentrations of ECx, EFs, or Dox alone & its combination. A combinational treatment yielded less cell viability compared to a single treatment ($p< 0.05$). Cytotoxicity was represented as percentage of MCF7-HER2 cells viability as the mean±SE of three values, *$p<0.05$.

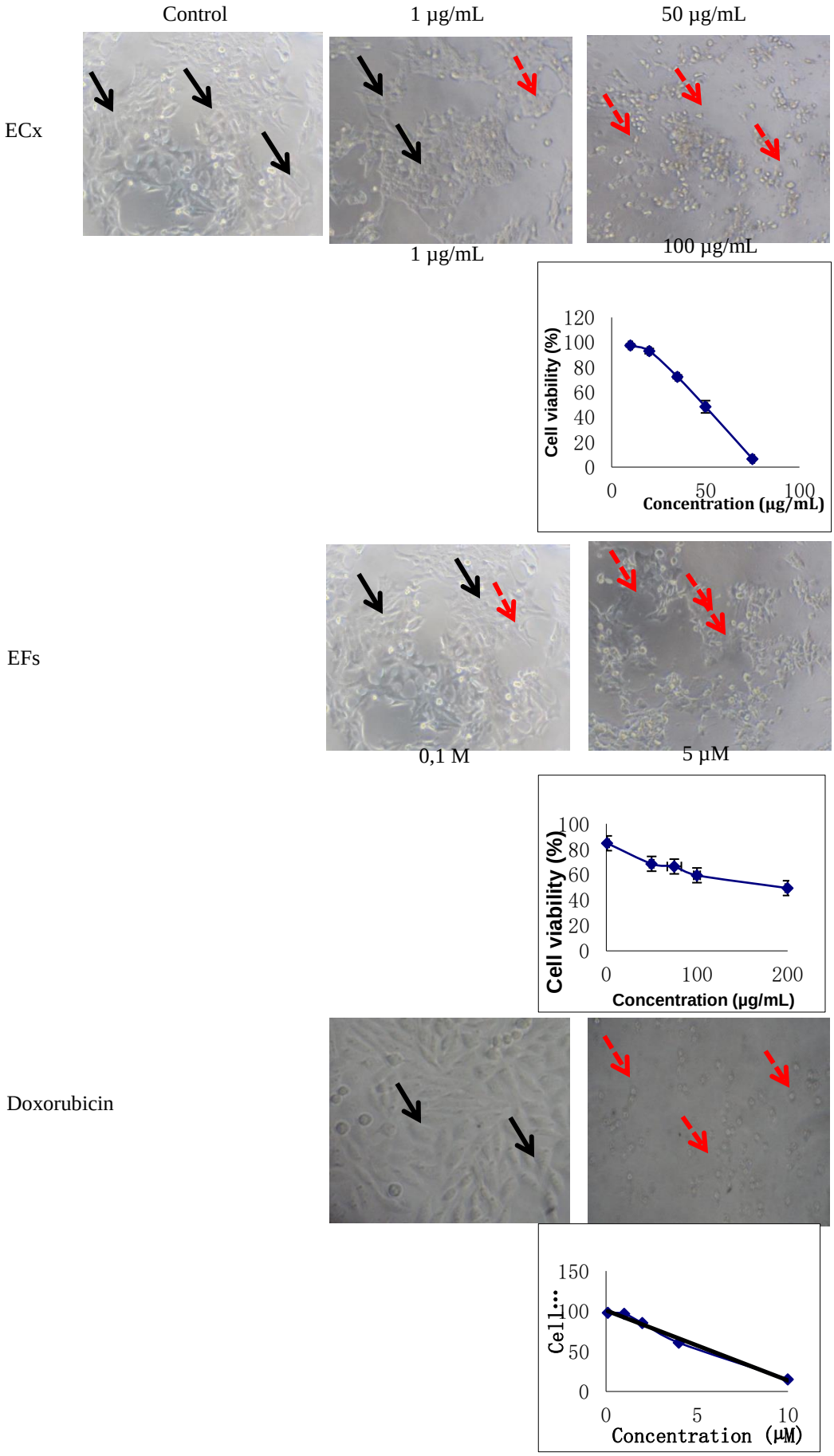


Figure 1.Cytotoxic effect of single treatment of ECx,EFs and doxorubicin on MCF7-HER2

The treatment was done by using low concentration (¼ IC_{50}) of each agent (45 µg/mL of ECx, 12 µg/mL of EFs, and 1.2 µM Dox). The combination of ECx, EFs and Dox resulted in a much greater inhibition of MMP-9 activity than a single treatment (figure 3).

DISCUSSION

The single treatment of ECx, EFs, and Dox is proven to have cytotoxic effect on MCF7-HER2 cells and its combination is synergy to reduce cell viability greater than a single treatment. It was supported by the previous study statements that each *Curcuma xanthorrhiza* and *Ficus septica* increased the effects of chemotherapy (Kunnumakkara et al., 2008).

Curcumin as the main active ingredient of *Curcuma xanthorrhiza*, inhibit the metastasis of breast cancer cells MDAMB-231 by suppressing the FAK pathway and lowering the expression of PI3K and were subsequently able to decrease the expression of VEGF (Lin et al., 2009). The decrease of VEGF will lead to the inhibition of the Ras-Raf-MEK-ERK pathway which reduce the activation of NF-κB so that transcription of the gene that encodes the proMMP-9 protein is also inhibited. Curcumin also inhibit β-catenin and reduce the loss of E-cadherin, which is related to the ability of invasion and metastasis of cancer cells (Thangapazham et al. 2006).

Similar to the mechanism of action of *Curcuma xanthorrhiza*, *Ficus sepica* also inhibit the activity of MMP-9. *Ficus sepica* suppress the activation of NF-κB and inhibits expression of COX-2 proteins that promotes invasion (Kim et al, 2010; Mandhare et al., 2015).

The interesting finding is that Dox as the main chemotherapy in advanced cancer, has the lowest antimigration activity compared to ECx and EFs. Bandyopadhyay et al. (2010) proved that doxorubicin increases migration and invasion of 4T1 and MDAMB-231cells through induction of TGFβ. Metastasis inhibition of doxorubicin which is seen in this study is due to cell death caused by doxorubicin treatment. Although the mechanism is not clear, single doxorubicin proved to inhibit the M5076 ovarian cancer cell metastasis invivo (Sugiyama and Sadzuka, 1999). ECx inhibits TGFβ (Kunnumakkara et al., 2008) in combination ECx-EFs-Dox, therefore the invasion induced by Dox via TGFβ can be eliminated as well as two others therapeutic agents work optimally in inhibiting cell metastasis.

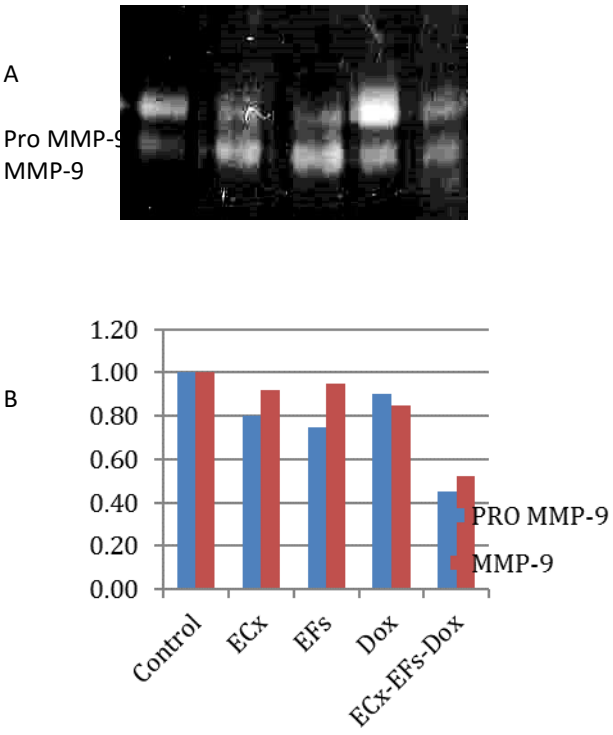


Figure 3. Effect of single and combination treatment of ECx,EFs and doxorubicin on activity of proMMP-9 and MMP-9. A. Band that shows pro MMP-9 & MMP-9 activities. B. Graph shows the relative intensity of pro MMP-9 & MMP-9 compared to controls (n=3).

CONCLUSIONS

ECx, EFs and doxorubicin solely revealed cytotoxic effect on MCF7-HER2 cells in a dose-dependent manner (IC50 ECx = 180 µg/mL, IC50 EFs = 48 µg/mL, and IC50 doxorubicin = 4.8 µM). The combination treatment of of ECx, EFs and doxorubicin on MCF7-HER2 cells showed a strong synergistic effect with CI = 0.30. Gelatin zymography analysis showed that combination of ECx, EFs and doxorubicin inhibited MMP-9 activity compared to single used. Combination of *Curcuma xanthorrhiza*, *Ficus septica* and doxorubicin may have potential to be developed as a combination for metastasis breast cancer treatment.

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