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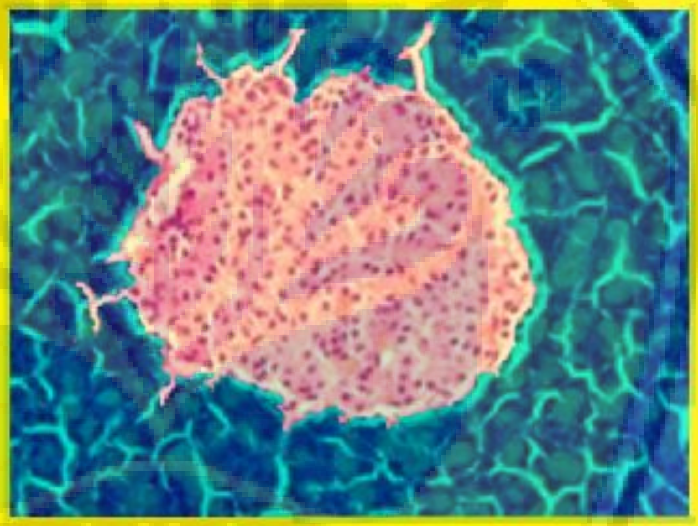

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In Vitro Study of the Combination of Doxorubicin, *Curcuma xanthorrhiza*, *Brucea javanica*, and *Ficus septica* as a Potential Novel Therapy for Metastatic Breast Cancer

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ABSTRACT

Less optimized therapeutic effects constrain the use of doxorubicin as the main agent of chemotherapy for metastatic breast cancer, resistance and side effects. Therefore we need a combination of more than one chemopreventive agent which has different molecular targets to solve that problem. The aims of this study is to prove the inhibitory effect of ethanolic extract of rhizome of *Curcuma xanthorrhiza* (ECx), fruit of *Brucea javanica* (EBj), leave of *Ficus septica* (EFs) and doxorubicin (Dox) alone and its combination on migration and invasion of a highly metastatic 4T1 breast cancer cell line. Cytotoxic activity of single and combination treatment was evaluated by MTT assay, followed by an experiment of apoptosis induction by using flow cytometry. The inhibitory effect on migration was observed by the scratch wound-healing assay. Furthermore, the observation of the activity of matrix metalloproteinase-9 (MMP-9) was analyzed by gelatin zymography. The results showed that ECx, EBj, EFs, and Dox has cytotoxic activity on 4T1 cells with the value of IC_{50} respectively 49.7±1.53 μg/mL, 59.9±1.79 μg/mL, 15.2±2.12 μg/mL and 1.2±0.23 μM. Furthermore, the combination of ECx-EBj-Dox and ECx-EBj-EFs revealed a synergistic effect on 4T1 cells and decrease cell viability through the induction of apoptosis and necrosis. Based on wound healing assay, 24 hours incubation of this combination inhibited 4T1 cells migration compared to single treatment. Gelatin zymography analysis showed that this combination also inhibited the activity of MMP-9 greater than a single use. *Curcuma xanthorrhiza*, *Brucea javanica*, and *Ficus septica* may have potential to be developed as a combination with or without doxorubicin for metastatic breast cancer treatment.

Keywords: *C xanthorrhiza*, *B javanica*, *F septica*, antimetastasis, 4T1 cells.

INTRODUCTION

Breast cancer is the first leading cause of cancer deaths in women. Doxorubicin is an important modality for the treatment of breast cancer metastasis in addition to surgery (Bapsy and Sahoo, 2006). Long term use of doxorubicin causes several side effects, resistance and toxicity to normal tissues (Smith *et al*., 2010). Therefore, combining doxorubicin with the chemopreventive agent is needed to increase the activity of doxorubicin, to overcome the drug resistance and to reduce its side effect (Sarkar and Li, 2006).

The chemopreventive agent used in this research is a combination of *Brucea javanica* (EBj), *Ficus septica* (EFs) and *Curcuma xanthorrhiza* (ECx). Many clinical studies have suggested that *Brucea javanica* can be used alone as a conventional treatment for various cancers. However, the present study shows that *Brucea javanica* has synergistic effects when combined with certain anticancer drugs or radiotherapy (Nie *et al*., 2012). The leaves of *Ficus septica* have been used to treat various cancers. The ethanolic extract of *Ficus septica* showed a cytotoxic effect on breast cancer T47D cell lines with an 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 nM) (Pratama *et al*., 2010). Besides, the extract induced apoptosis and downregulated the expression of Bcl-2 protein in breast cancer cells MCF-7 (Sekti *et al*., 2010). The principal components of *Curcuma xanthorrhiza* are curcumin and xanthorrhizol. Curcumin suppresses many 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.

There have been several reports about the combination of doxorubicin with one kind of chemopreventive agent, and its synergistic has been proved (Lewandowska *et al*., 2014), but study using a combination of more than one kind of chemopreventive agents with doxorubicin has not been done, mainly to prove its effectiveness as an antimetastasis agent in advanced breast cancer. This research combined ECx, and EBj with Dox to increase effectivity, to overcome drug resistance, and to reduce its side effect while the combination of three chemopreventive agents (ECx, EFs and EBj) without chemotherapy Dox is assumed to get nontoxic nature of EFs and to eliminate the side effect of Dox.

MATERIAL AND METHODS

The rhizome of *Curcuma xanthorrhiza*, the fruit of *Brucea javanica* and the leave of *Ficus septica* was macerated with 70% ethanol. The procedure was done at Medicinal Plant and Traditional Medicine, Research and Development Centre (B2P2TO-OT) Tawangmangu, Indonesia. Doxorubicin was obtained from Sigma. A DMSO (Merck) solution was used to dilute ethanolic extract of ECx, EFs and EBj. The final DMSO concentration was set at less than 1%.

Cells culture

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), penicillin-streptomycin 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

Various concentrations of samples (ECx, EBj, EFs, Dox, and its combination), DMEM culture medium (Gibco) with 10% FBS, 1% penicillin-streptomycin and fungizone were used to conduct the cytotoxic colorimetric MTT assay on 4T1 breast cancer cells. It was used to determine the IC_{50} value. Afterwards, a cytotoxicity assay was also conducted to determine the effect of the combination of various sample concentrations treatment (Mosmann, 1983).

Flow cytometric apoptosis assay

Cells were added with Annexin-V-FLUOS Staining Kit (Roche) consisting of 200 µL of binding buffer, 2 µL of Annexin V, 2 µL propidium iodide (PI) and incubated for 10 minutes in the dark room according to manufacturer's instruction, then transferred into a flow cytometry tube and analyzed by flow cytometer (BD FACS Calibur, BD Bioscience) (Engeland *et al*., 1998).

Scratch wound healing assay

The 4T1 cells (7.5×10^4) were cultured in 24-well plate and incubated for 24h. After starvation, cells were scratched by using sterile yellow tip then treated with various concentrations of samples. The cells were documented at 0, 18, and 24h (Liang *et al.,* 2007). The results were analyzed by using ImageJ software and converted to percentage closure parameter.

Gelatin zymography

The SDS-PAGE 8% supplemented with 0.1% gelatin was used to determine the activity of MMP-9 in the culture medium. After electro-phoresis running, gels were washed and incubated at room temperature. The reaction buffer was added and incubated for 24h at 37 °C. Gels were stained by using Coomassie Brilliant Blue R-250 (Sigma) and

destained 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. A statistically significant difference was considered to be present at $p<0.05$. The results of scratch wound healing assay and gelatine zymography were documented and analyzed using Image J software before ANOVA.

Combination cytotoxicity assay and synergicity

The effect of combination treatment on 4T1 cells was evaluated by calculating the Combination Index (CI) value using the formula. Interpretation of the data was based on the classification (Reynolds and Maurer, 2005).

RESULTS AND DISCUSSION

Effects of ECx, EBj, EFs, Dox alone and its combination on cell viability of 4T1

A single treatment of ECx, EBj, EFs, or Dox showed cytotoxic effect on 4Tl breast cancer cells with IC_{50} value respectively 49.7±1.53 μg/mL, 59.9±1.79 μg/mL, 15.2±2.12 μg/mL and 1.2±0.23 μM (Figure 1). The reduction of cell viability of combination of ECx-EBj-EFs and ECx-EBj-Dox is greater than the single treatment. The combination of ECx-EBj-Dox using concentrations of ½ IC_{50} showed synergistic effects with a CI value of 0.63 and reduced cell viability up to 65% (Sutejo, 2015). The combination of ECx-EBj-EFs using concentrations of ½ IC_{50} showed synergistic effects with a CI value of 0.83 and reduced cell viability up to 63% (Figure 2).

The combination of ECx-EBj-EFs and ECx-EBj-Dox increase apoptosis and necrosis on 4T1 cells

The observation of apoptosis was done by using concentration ⅓IC_{50} of each agent. All single treatments and combination induced apoptosis on 4T1. The percentage of living cells in control was 92.18%, while the percentage of apoptotic cells is 6.34% and necrotic cells were 2.17%. After a single treatment ECx, EBj, EFs, and Dox, the percentages of cells undergoing apoptosis were respectively 12.08%, 6.22%, 10.47% and 13.41%. The treatment with ECx-EBj-EFs and ECx-EBj-Dox for 24h incubation increased the apoptotic cells by 14.46% and 11.99% (Figure 3).

The combination of ECx-EBj-EFs and ECx-EBj-Dox inhibit migration of 4T1 cells

The observation of cell migration activity was done by using concentration ¼ IC_{50} of each agent. The effect on cell migration was observed at time point 0, 18, and 24h. The single treatment of ECx, EBj, EFs, Dox and its combination decrease cell migration of 4T1. The ability of the most robust inhibition to the lowest showed by the combination of ECx-EBj-EFs, ECx-EBj-Dox, and then ECx, EFs, followed by EBj, and the least one was doxorubicin (Figure 4).

The combination of ECx-EBj-EFs and ECx-EBj-Dox inhibit activity of MMP-9 on 4T1 cells

The treatment was done by using concentration ¼ IC_{50} of each agent. The single treatment of ECx, EFs, and EBj is more potential to inhibit the activity of MMP-9 than doxorubicin. The combination of ECx-EBj-EFs and ECx-EBj-Dox resulted in much greater inhibition of MMP-9 activity than a single treatment (Figure 5).

Discussion

The single treatment of ECx, EBj, EFs, and Dox was proven to have a cytotoxic effect on 4T1 cells (Figure 1), and its combination showed a synergism to reduce cell viability greater than a single treatment (Figure 2). It was supported by the previous study statements that each *Curcuma xanthorrhiza*, *Brucea javanica*, and *Ficus septica* increased the effects of chemotherapy (Kunnumakkara *et al*., 2008). Combination of ECx-EBj-EFs was as potent as ECx-EBj-Dox to induce 4T1 cells death (Figure 3). Mechanism of cell growth inhibition both two combinations was due to apoptosis and necrosis pathways. The molecular mechanisms underlined the apoptosis after treatment of ECx, EBj and EFs in 4T1 cells was through p53-independent pathways, due to 4T1 breast cancer cells line characteristic which is lack of protein P53 expression (Tao *et al*., 2008). Whereas doxorubicin induces necrosis through increased TNFα and ROS (Sugimoto *et al*., 2002).

The rhizomes of *Curcuma xanthorrhiza* contains volatile oil, saponin, flavonoid, and tannin. Chemistry analysis showed that the main substances of *Curcuma xanthorrhiza* are curcumin, desmethoxycurcumin and xanthorrhizol (Choi *et al*., 2005). Most of the studies on *Ficus septica* leaves reported on the phenanthroindolizidine

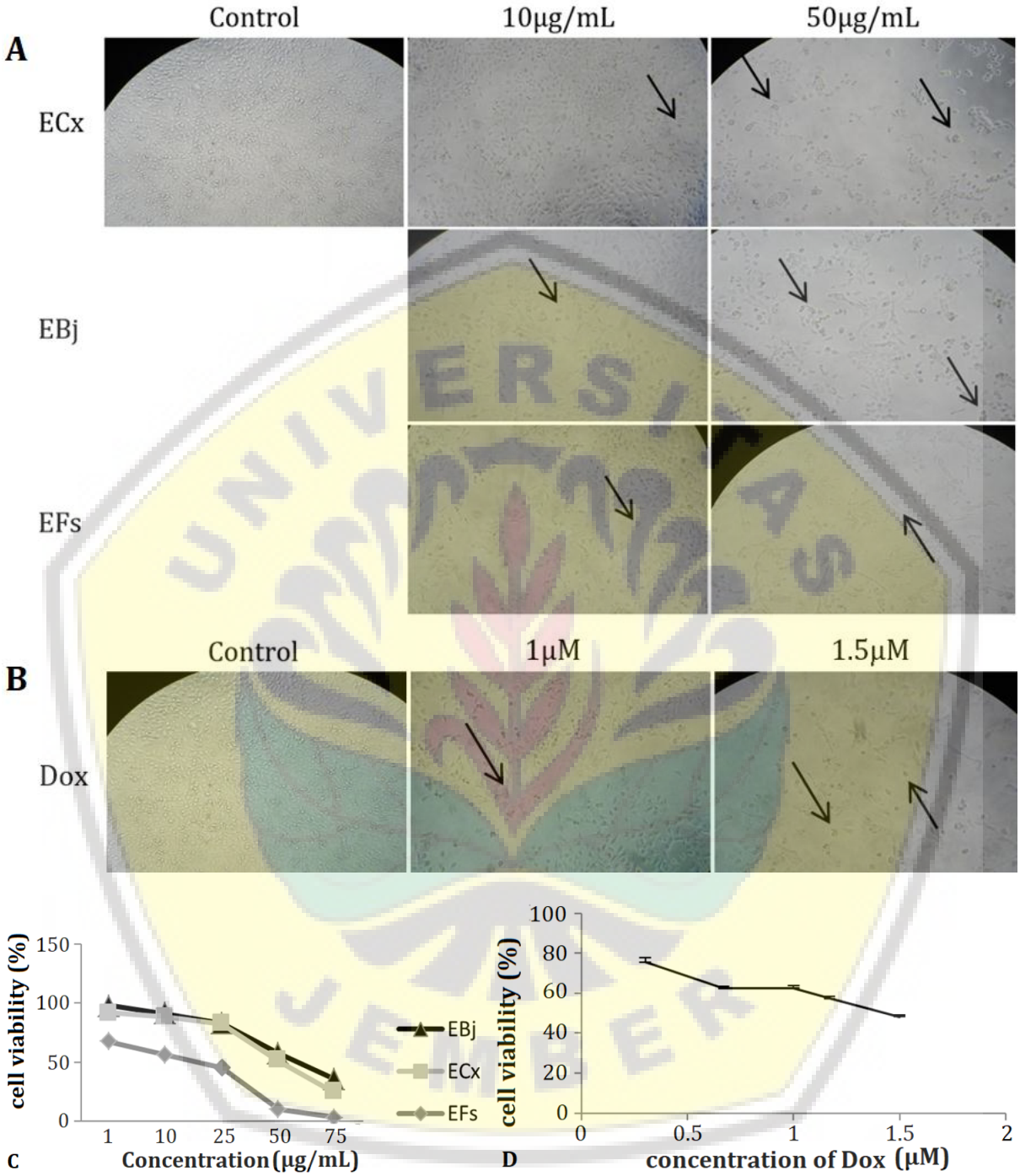
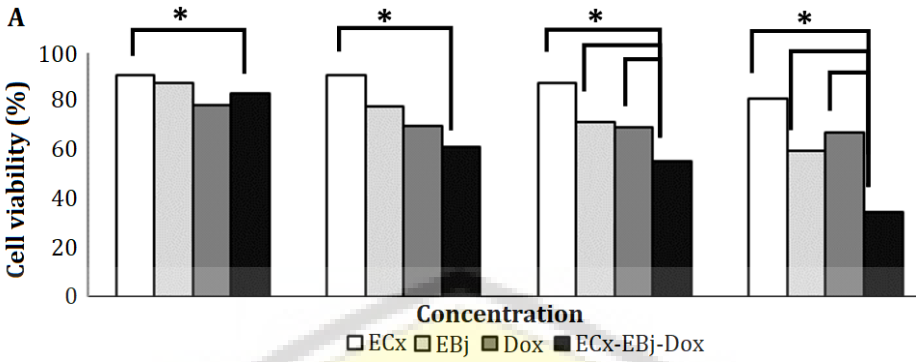
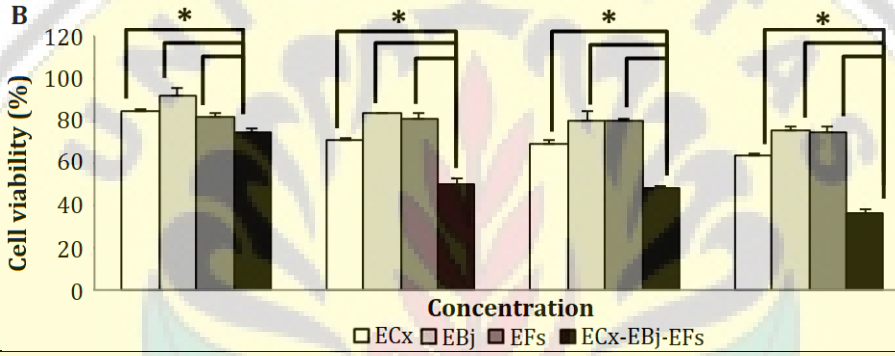


Figure 1. Cytotoxic effect of single treatment of ECx, EBj, EFs, and Dox on 4T1 Cells. $5x10^3$ cells/well were seeded in 96 well plate and incubated for 24h, then treated with ECx, EBj, EFs and Dox. Cell viability was determined by using MTT assay as described in the method. Morphology cells after a single treatment of ECx, EBj, EFs (A) or Dox (B) for 24h. Arrows indicate cell morphological changes. Cell viability profile after a single treatment of ECx, EBj, EFs (C) or Dox (D) for 24h. Profile of cell viability were means ± SD from 3 independent experiments.



	1/6 IC_{50}			1/4 IC_{50}			1/3 IC_{50}			½ IC_{50}		
ECx (µg/mL)	8.3	+	+	12.5	+	+	16.7	+	+	25	+	+
EBj (µg/mL)	10	+	+	15	+	+	20	+	+	30	+	+
Dox (µM)	0.2	+	+	0.3	+	+	0.4	+	+	0.6	+	+



	1/6 IC_{50}			1/4 IC_{50}			1/3 IC_{50}			½ IC_{50}		
ECx (µg/mL)	8.3	+	+	12.5	+	+	16.7	+	+	25	+	+
EBj (µg/mL)	10	+	+	15	+	+	20	+	+	30	+	+
EFs (µg/mL)	2.5	+	+	3.75	+	+	5	+	+	7.5	+	+

Figure 2. Combination effect of ECx, EBj, EFs, and Dox on the viability of 4T1 cells. $5x10^3$ cells per well were incubated for 24h and exposed with various concentrations of (A) ECx, EBj, or Dox alone and its combination, (B) ECx, EBj, or EFs alone and its combination, then subjected for MTT assay. A combinational treatment yielded less cell viability compared to a single treatment (p 0.05). Cytotoxicity was represented as a percentage of 4T1 cells viability as the mean±SE of three values.

alkaloid which has a cytotoxic effect on cancer cells (Sekti *et al.*, 2010). Several natural components from *Brucea javanica* fruit include the tetracyclic triterpene quassinoids, anthraquinone, olein, oleic acid, linoleic acid, pregnane glucosides, and sesquiterpenes. In particular, tetracyclic triterpene quassinoids (brucein A and bruceantin) are the main active ingredients of *Brucea javanica* with remarkable antitumor activity (Chen *et al.*, 2013).

The treatment of ECx, EBj, EFs, and Dox alone or in combination inhibited the migration of 4T1 breast cancer cells (Figure 4). The interesting finding was that Dox as the main chemotherapy in advanced cancer has the lowest antimigration activity compared to ECx, EBj and EFs (Figure 4). Bandyopadhyay *et al.* (2010) and Krstic and Santibanez (2014) proved that doxorubicin increases migration and invasion of 4T1 and MDAMB-231cells through induction of TGFβ. Those findings in line with our result that the combination of ECx-EBj-EFs inhibited cells migration greater than ECx-EBj-Dox. Metastasis inhibition of doxorubicin which was seen in this study was 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 *in vivo* (Sugiyama and Sadzuka, 1999).

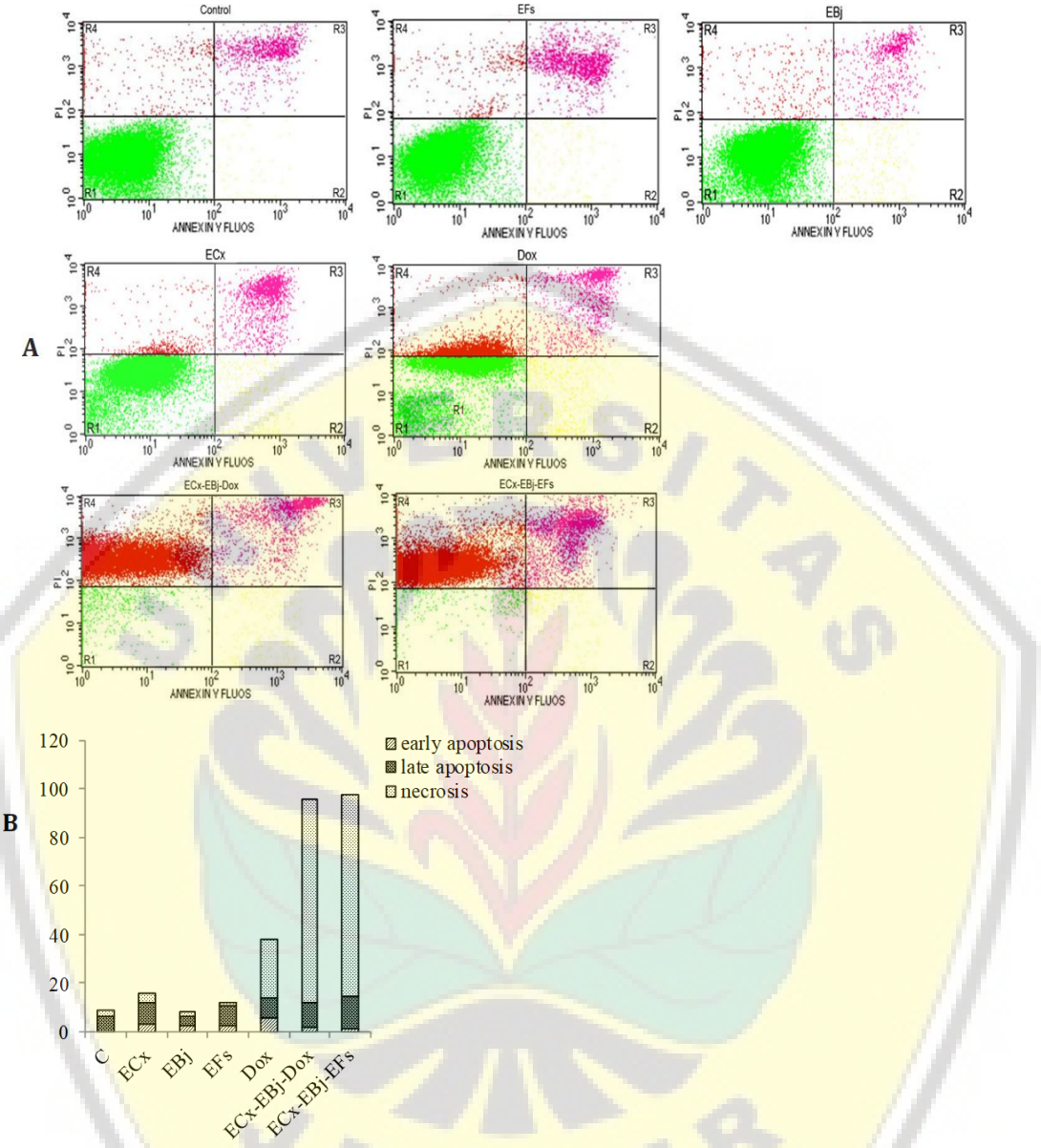


Figure 3. Apoptosis effects of ECx, EBj, EFs, and Dox single and its combination on 4T1. Cells were harvested after treatment of 16.7 µg/mL of ECx, 20 µg/mL of EBj, 5 µg/mL EFs and 0.4 µM Dox for 24h single or in combination, then stained using Annexin V-FITC/PI and were analyzed by using flow cytometry. (A) The distribution profiles of living cells (R1), early apoptosis (R2), late apoptosis (R3), and necrosis (R4), (B) quantification of cells undergoing early apoptosis, late apoptosis and necrosis.

Several reports related to the anti-metastasis potency of the active compounds contained in the extracts we used are as follows. Curcumin as the main active ingredient of ECx, inhibit the migration 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). Curcumin also inhibits the expression of MMP-9 causing a decrease of MMP-9 activity, inhibits 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). Alkaloids phenanthro-indolizidine from *Ficus septica* inhibits expression of COX-2 proteins (Mandhare *et al*., 2015).

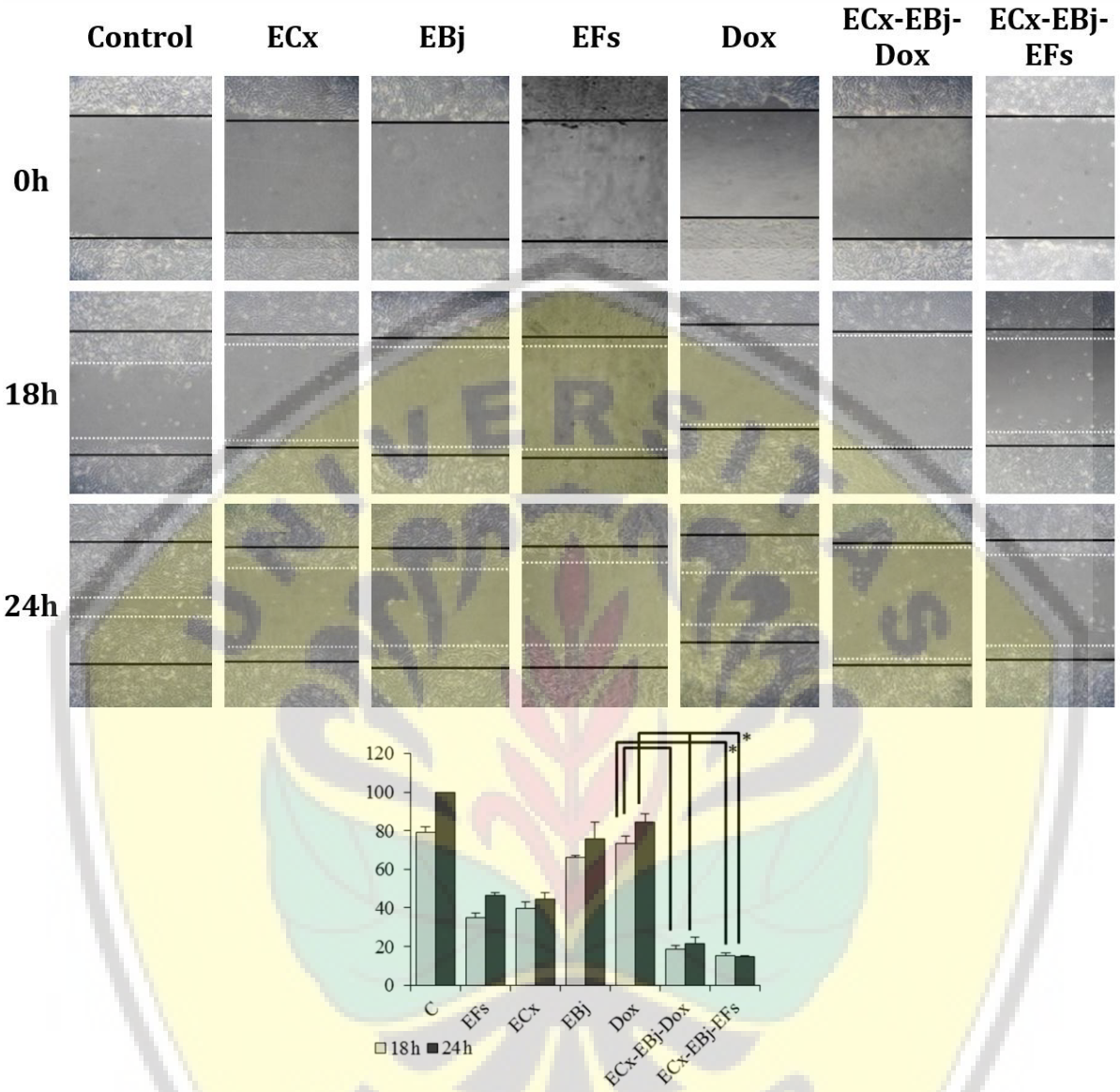


Figure 4. Effects of ECx, EBj, EFs, and Dox alone and its combination on 4T1 cell migration. (A) The morphology of the cells after the scratch and treated with 12.5 µg/mL ECx; 15 µg/mL EBj, 3.75 µg/mL EFs, or 0.3 µM Dox single and its combination. The observation was made after 0, 18, 24, and 42h of treatment under an inverted microscope with 100x magnification. (B) The percentage of 4T1 closure after 18h and 24h of treatment. The value was a means of % closure ± SD from 3 independent experiments. The area of the scratch was analyzed using ImageJ software then % closure was calculated following the procedure of the analysis. The asterisk (*) indicates differences ($p<0.95$; n=3)

Brucein A and bruceantin are known to inhibit the invasion and migration of tumour cells targeting at MRP-1/CD9 and integrinα-5 (Nan *et al*., 2015). Brucein A from *Brucea javanica* lowered VEGF expression so that no signal is transmitted through the VEGF receptor (Xinjie and Linyi, 2013). Moreover, *Brucea javanica* and *Ficus septica* also suppress the activation of NF-κB and inhibits expression of COX-2 proteins that promote invasion (Kim *et al*., 2010; Mandhare *et al*., 2015). The mechanisms above explain our findings that the combination of ECx-EBj-EFs and ECx-EBj-Dox inhibited cells migration greater than the single treatment (Figure 4B).

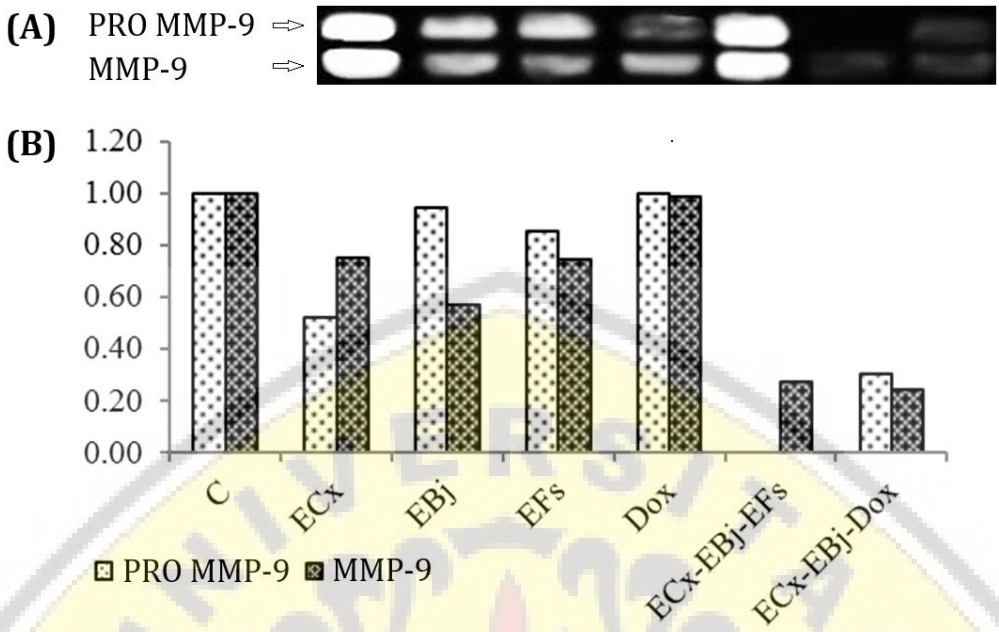


Figure 5. Effect of ECx, EBj, EFs, Dox alone & its combination in MMP-9 activity on 4T1 cells. Cells were treated with 12.5 µg/mL ECx; 15 µg/mL EBj, 3.75 µg/mL of EFs, or 0.3 µM Dox and their combination. MMP-9 activity assay was conducted using gelatin zymography according to the method. Analysis of the results was done by using ImageJ software to measure the intensity of gelatin degradation by pro-MMP-9 &MMP-9 in the gel. (A) The bands that showed the pro MMP-9 & MMP-9 activities. (B) The graph of the relative intensity of pro-MMP-9 & MMP-9 compared to controls (n=3).

The fact that Dox has not only induced cell migration but also cell invasion also explains our data that the combination of ECx-EBj-EFs inhibited MMP-9 activity greater than ECx-EBj-Dox (Figure 5B).

CONCLUSION

The results of this study conclude that both two combinations of ECx-EBj-Dox and ECx-EBj-EFs have a cytotoxic effect via apoptosis and necrosis pathways. The combination of ECx-EBj-EFs has antimigration activity and inhibition of cancer cell invasion greater than ECx-EBj-Dox. Both two combinations have a potency to be developed as a co-chemotherapy agent in breast cancer metastasis. Nevertheless, further researches are still needed in order to apply such combination therapy for cancer patients.

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