JURNAL ILMU KEFARMASIAN INDONESIA, April 2009, hal. 13-18
ISSN 1693-1831

Vol. 7, No. 1

Synergistic Effect of *Areca catechu* L. Ethanolic Extract and Its Chloroform Fraction with Doxorubicin on MCF7

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Accepted June 18th 2008, Approved March 17th 2009

Abstrak: Ekstrak etanol biji buah pinang (*Areca catechu* L.) menunjukkan efek sitotoksik pada sel kanker MCF7 dan WiDr. Penelitian ini bertujuan untuk mempelajari efek sinergisme antara ekstrak etanol biji buah pinang (AE) dan fraksi kloroformnya (ACF) dengan doxorubicin (Dox) dalam pemacuan apoptosis sel MCF7. Ekstrak etanol dipartisi dengan *n*-heksan dan kloroform untuk mendapatkan fraksi kloroform. Efek sitotoksik AE, ACF, dan Dox pada perlakuan tunggal dan kombinasi ditentukan dengan metode MTT. Pengamatan apoptosis dilakukan dengan pengecatan DNA dengan akridin oranye-etidium bromida (*double staining*). Imunositokimia dilakukan untuk melihat ekspresi COX-2 dan Bax. Kombinasi Dox 100, 250, 334, dan 500 nM dengan AE 8 µg/ml; Dox 100 nM dengan AE 20 µg/ml; serta Dox 100 dan 250 nM dengan ACF 7 dan 18 µg/ml memperlihatkan efek sinergistis yang kuat (CI 0,1–0,3). Sementara itu, kombinasi Dox 250, 334, dan 500 nM dengan AE 20, 27, dan 40 µg/ml; Dox 100 nM dengan AE 27 dan 40 µg/ml; Dox 100 nM dengan AE 20 µg/ml; serta 500 nM dengan ACF 24 dan 35 µg/ml menunjukkan efek sinergistis (CI 0,3–0,7). Secara keseluruhan, kombinasi AE dan ACF dengan Dox memperlihatkan efek sinergistis pada MCF7 dengan indeks kombinasi (CI) kurang dari 0,9 ($P<0,05$). Perlakuan kombinasi juga memacu apoptosis. Penekanan ekspresi Bcl-2 terjadi pada perlakuan kombinasi ACF-Dox. Hasil penelitian ini menunjukkan bahwa kombinasi AE dan ACF dengan Dox memberikan efek sinergistis dalam pemacuan apoptosis dengan kemungkinan mekanisme melalui penekanan ekspresi Bcl-2.

Kata kunci: *Areca catechu*, kombinasi, sel MCF7, apoptosis, Bcl-2.

INTRODUCTION

CELL cycle arrest and apoptosis induction are two of the prospective strategies on cancer therapy[1]. The cells are arrested to repair any damages which occur during cell cycle. Cells undergo apoptosis omitting the abnormal cells in order to maintain the balance of cell growth and the other normal biological processes[2]. The capabilities of the chemotherapeutic agents to induce cell cycle arrest and apoptosis on cancer cells determine its potency as cytotoxic agent[3].

Doxorubicin is one of chemotherapeutic agent which commonly used in breast cancer therapy[4]. Doxorubicin performs high capability to induce apoptosis[5]. However, doxorubicin administration caused various side effects, such as myelosuppression, immunesuppression, and cardiac toxicity[6,7]. Doxorubicin also induces cancer cell resistance through activation of *multidrug resistance* (MDR) gene leading to the decrease of cancer cell sensitivity against doxorubicin. Therefore, the finding of agents which increase cancer cell sensitivity is important to give more effective treatment and reduce side effects.

Previous study showed that *A. catechu* ethanolic extract and its chloroform fraction inhibit cell growth and induce apoptosis on MCF7 and WiDr cells[8,9,10]. Moreover, Areca chloroform fraction (ACF) performs apoptosis induction by suppressing of Bcl-2 expression on MCF7[9], a chemotherapeutic resistant cell line[11,12]. The ACF possibly increases the sensitivity of resistant cancer cells against chemotherapeutic agent.

MATERIALS AND METHODS

MATERIALS. Areca seeds were obtained from Balai Penelitian Tanaman Obat (BPTO), Indonesia.

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Areca seeds ethanolic extract (AE) was prepared by extraction using ethanol. The chloroform fraction (ACF) was prepared by extracting AE using *n*-hexane and chloroform. MCF7 cell line obtained from Prof. Tatsuo Takeya (*Nara Institute of Science and Technology*, Japan) was cultured in DMEM medium (Gibco) with 10% Fetal Bovine Serum (Gibco) and 1% Penicillin-Streptomycin (Gibco). Monoclonal antibody anti-Bcl-2 was purchased from Dako, hydrochloric acid (HCl) and isopropanol were purchased from Merck, dimethylsulfoxide (DMSO), MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide], and other chemicals were purchased from Sigma.

METHODS. Cell viability assay. Cell viability was determined by using MTT assay. MCF7 cells ($3x10^3$ cells/well) were distributed onto 96 well-plates and incubated at 37°C for 48 hours. Serial dilution of the doxorubicin to calculate IC_{50} were used at 100–1000 nM . Serial dilution of the AE, ACF and Doxorubicin (Dox) to calculate CI were used at 1/10, ¼, 1/3, and ½ of IC_{50}. After rinsed the cells using phosphate buffer saline (PBS), cells were treated with the samples and after 48 hours incubation MTT reagent was added. Stopper reagent (hydrochloric acid 4N–isopropanol, 1:100) was added after formazan formation prior to MTT reduction. The absorbance of each well was measured using ELISA reader (Bio-Rad) at 595 nm.

Apoptosis assay. After 48 hours incubation at 37°C, MCF7 cells ($5x10^4$ cells/well) were treated with AE (60 µg/ml), ACF (60 µg/ml), Dox (500 nM) and combination of Dox-AE and Dox-ACF for 48 hours. After incubation, cells were stained for 5 minutes with acrydine orange–ethydium bromide staining solution (each 5 µg/ml in PBS) and viewed immediately by fluorescence microscope (Zeiss MC 80). Apoptotic cells which had lost their membrane integrity appeared orange and showed morphological features of apoptosis. Cells were identified as apoptotic on the basis of specific morphological criteria, including condensation and fragmentation of chromatin, and formation of apoptotic bodies.

Immunocytochemistry assay. MCF7 cells ($5x10^4$ cells/well) were seeded onto coverslip in 24 well-plates. Following 48 hours incubation at 37°C, cells were treated with ACF (60 µg/ml), Dox (500 nM) and combination of Dox-ACF for 15 hours. The cells attached on coverslip's surface were been treated with immunocytochemistry reagent according to the manufacturer's protocol. Cells were observed by using light microscope (Zeiss). The brown color of cytoplasm indicates positive expression while blue/violet indicates negative expression.

Statistical analysis. The absorbances were converted into percentage of cell viabilities and analyzed by using Excell MS Office 2003 and probit analysis (SPSS 11.5) to obtain IC_{50} value. One-way Anova was used to assess the significance among the treatment ($P<0.05$) and post-hoc test was done using Tukey's Significant Differences test.

Concentration ratio of combinatorial treatment was used under IC_{50} (Table 1). The cell viabilities of the combinations were used to determine *Combination Index* (CI) using the following equation:

$$CI= (D)_1/(Dx)_1 + (D)_2/(Dx)_2$$

D is the applied concentration and Dx is the calculating concentration which inhibits "x" percent of cell growths in combinatorial treatments. The various degrees of synergism or antagonism have been interpreted by using CI values (Table 2)[13].

In apoptosis observation, at least 100 cells were evaluated to determine the percentage of apoptosis induction.

Table 1. Concentration ratio of the combination treatment.

Doxorubicin (IC_{50})	AE or ACF (IC_{50})			
	1/10	¼	1/3	½
1/10	1/10 : 1/10	1/10 : ¼	1/10 : 1/3	1/10 : ½
¼	¼ : 1/10	¼ : ¼	¼ : 1/3	¼ : ½
1/3	1/10 : 1/3	1/3 : ¼	1/3 : 1/3	1/3 : ½
½	1/10 : ½	½ : ¼	½ : 1/3	½ : ½

Table 2. Interpretation of CI values[13]

CI	Interpretation	CI	Interpretation
<0.1	very strong synergism	0.9–1.1	nearly additive
0.1–0.3	strong synergism	1.1–1.45	slight to moderate antagonism
0.3–0.7	synergism	1.45–3.3	antagonism
0.7–0.9	moderate to slight synergism	>3.3	strong to very strong antagonism

RESULTS AND DISCUSSIONS

Synergistic effect of AE and ACF with Dox. The result showed that cytotoxic effect represented in IC_{50} value of Dox on MCF7 cell was 957 nM (data not shown). Previous study showed that IC_{50} values of AE and ACF are 75µg/ml and 65µg/ml

respectively[9]. Both of them are potential to increase MCF7 sensitivity against dox, the commonly used chemotherapeutic agent on breast cancer treatment. Furthermore, calculation of synergistic effect of Dox-AE and Dox-ACF with CI parameter showed that all of the combination performed synergistic effects (CI<0.9). Combination of AE (8, 20, 27 and 40 µg/ml) and ACF (7, 18, 24 and 35 µg/ml) with Dox (100, 250, 334, and 500 nM) inhibited MCF7 cells growth compared to single application in the same concentration (Figure 1A and 2A).

Based on the result, combination of 100, 250, 334, and 500 nM Dox with 8 µg/ml AE; and 100 nM Dox with 20 µg/ml AE showed strong synergistic effect (CI 0.1–0.3). Combination of 250, 334, and 500 nM Dox with 20, 27 and 40 µg/ml AE; 100 nM Dox with 27 and 40 µg/ml AE; and 100 nM Dox with 20 µg/ml AE showed synergistic effect (CI 0.3–0.7) (Figure 1B; Table 3). The results suggested that Dox-AE performed synergistic effect and indicated a better efficacy compared to the single application.

In Dox-ACF treatment, combination of 100 and 250 nM Dox with 7 and 18 µg/ml ACF showed strong synergistic effect (CI 0.1-0.3). Moreover, combination of 100, 250, 334, and 500 nM Dox with 24 and 35 µg/ml ACF showed synergistic effect (CI 0.3-0.7) (Figure 2B; Table 4). Taken together, the combination performed synergistic effect on MCF7 cells so that it is promising on combinatorial therapy.

Apoptotic effect of AE and ACF in combination with Dox. AE or ACF suggested to increase cells sensitivity against Dox. Apoptosis study showed that the Dox-AE and Dox-ACF combination increased apoptosis induction (Figure 3E-F) compared to the single application (Figure 3B-D). Apoptosis quantitative data showed that AE, ACF, Dox-AE and Dox-ACF treatment induced apoptosis on MCF7 cells 10.24, 17.89, 7.76, 23.29, and 23.47% respectively (Table 5).

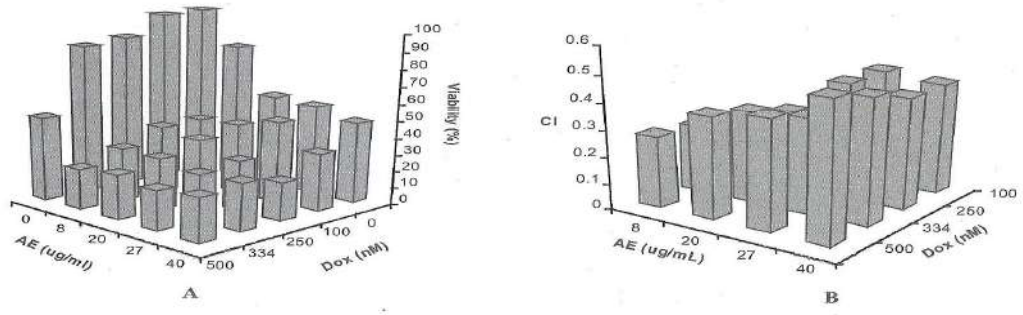

Figure 1. Combinatorial effect of Dox-AE on cell growth inhibition. (A) Combination of AE (8–40 µg/mL) and Dox (100-500 nM) on cell viability. (B) Synergistic effect of Dox-AE with CI<0.9. Combination Index (CI) was calculated as concentration ratio in single and combination treatments.

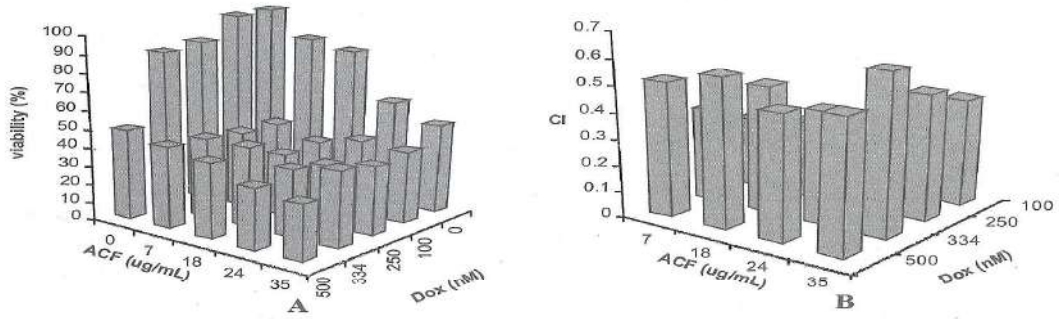

Figure 2. Combinatorial effect of Dox-ACF on cell growth inhibition. (A) Combination of ACF (7–35 µg/mL) and Dox (100–500 nM) on cell viability. (B) Synergistic effect of Dox-ACF with CI<0.9. Combination Index (CI) was calculated as concentration ratio in single and combination treatments.

Table 3. CI values of Dox-AE combination.

AE concentration (µg/mL)	Dox concentration (nM)			
	100	250	334	500
8	0.15	0.26	0.25	0.27
20	0.29	0.35	0.34	0.38
27	0.43	0.34	0.36	0.41
40	0.42	0.37	0.47	0.51

Table 4. CI values of Dox-ACF combination.

ACF concentration (µg/mL)	Dox concentration (nM)			
	100	250	334	500
7	0.15	0.27	0.35	0.51
18	0.22	0.29	0.48	0.56
24	0.32	0.33	0.43	0.47
35	0.41	0.48	0.61	0.50

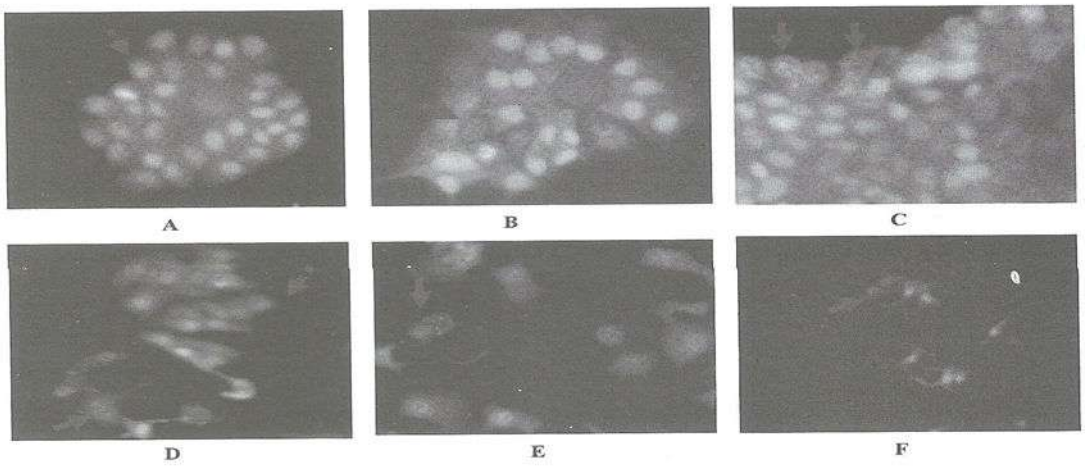

Figure 3. Dox-AE and Dox-ACF combination induced apoptosis on MCF7 cells. MCF7 cells (5x104) treated with AE (60 µg/mL), ACF (60 µg/mL) and Dox (500 nM) in single and combination for 48 h. (A) control cells, cells treated with (B) 500 nM Dox, (C) 50 µg/mL AE, (D) 35µg/mL ACF, (E) Dox-AE (500 nM-50 µg/mL), and (F) Dox-ACF (500 nM-35 µg/mL). Cells were stained with acrydine orange-ethidium bromide and observed by using fluorosence microscope, 100x magnification, ⟶ apoptosis, ⇢ viable cells.

Table 5. Apoptotic effects of Dox-AE and Dox-ACF against MCF7 cells.

No	Treatment	Apoptosis (%)*)
1	Untreated cells	0
2	Dox 500 nM	7.76
3	AE 50 µg/mL	10.24
4	Dox-AE	23.29
5	ACF 35 µg/mL	17.89
6	Dox-ACF	23.47

Dox-ACF combination suppressed Bcl-2 expression. The result showed that 35 µg/ml ACF seemingly suppressed Bcl-2 expression on MCF7 cells (Figure 4D) and 500nM Dox did not significantly suppress Bcl-2 expression compared to control cells (Figure 4C). The combination of Dox-ACF highly suppressed Bcl-2 expression, significantly different to control cells (Figure 4E). These findings indicated that ACF may contribute on enhancement of Dox cytotoxicity through suppressing of Bcl-2 expression, leading to apoptosis induction.

DISCUSSIONS

MCF7 cell line is a resistant cell against several chemotherapeutic agent[11,12]. It is interesting that both Dox-AE and Dox-ACF combination showed synergistic effect on MCF7 cells (CI<0.9). This finding suggested that AE or ACF were promising to be combined with doxorubicin for breast cancer treatment. Apoptosis study showed that combination of Dox-AE and Dox-ACF induced apoptosis stronger than the single application. Furthermore, we investigated the possible mechanism mediated apoptosis induction of the combination.

MCF7 cell line overexpresses an antiapoptotic Bcl-2 protein[14,15]. Therefore, suppression of Bcl-2 expression by ACF on MCF7 cells (Figure 4) is a prospective result for cancer treatment. Mechanism of *p53* dependent-apoptosis begins from the cellular stress followed by *p53* tumor supressor gene expression. Expression of *p53* induces proapoptosis protein (such as Bad, Bax, and Bid) expression and cytochrome C release. This mechanism is inhibited by Bcl-2 which is expressed through the NFκB-transcriptional activation[16].

Flavonoid compounds in AE/ACF possibly mediate the inhibition of NFκB activation. Flavonoids are potential inhibitor of protein kinases, such as IKK, which is important for NFκB activation[17]. On the other hand, Dox induces DNA damage and followed by Fas-L expression. Fas-L in complex with Fas receptor activates caspase 8 leading to Bid activation to form tBid and followed by Bax

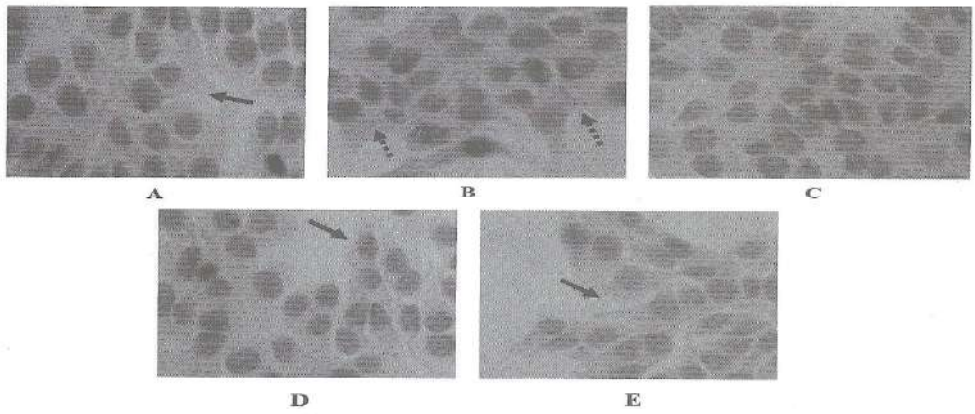

Figure 4. Bcl-2 expression after Dox-ACF treatment. MCF7 cells (5 X 104) treated with ACF (35 µg/mL) and Dox (500 nM) in single and combination for 15 h. (A) cells without Bcl-2 antibody, (B) control cells, cells treated with (C) 500 nM Dox, (D) 35 µg/mL ACF, and (E) Dox-ACF (500 nM-35 µg/mL), 400x magnification, - - ► positive, ——► negative.

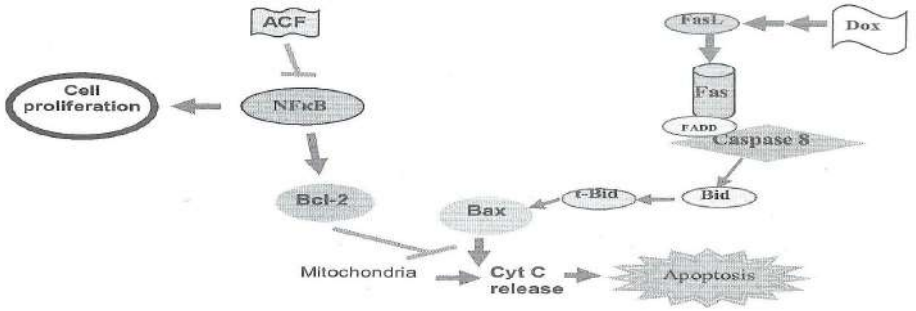

Figure 5. Possible synergistic mechanism of Dox-ACF inducing apoptosis. Dox induces DNA damage and followed by Fas-L expression. Fas-L in complex with Fas receptor activates caspase 8 leading to Bid activation to form tBid and followed by Bax localization on mitochondria outer membrane and increase cytochrome C release and then induce apoptosis. Suppression of Bcl-2 expression by ACF may increase sensitivity of MCF7 cells against doxorubicin on apoptosis induction.

localization on mitochondria outer membrane and increase cytochrome C release and then induce apoptosis. Suppression of Bcl-2 expression by ACF may increase sensitivity of MCF7 cells against doxorubicin on apoptosis induction (Figure 5).

CONCLUSIONS

The result showed that AE and ACF elevated sensitivity of MCF7 cells against doxorubicin. The combination performed synergistic effect to induce apoptosis. The research exhibited the relationship of synergistic effect of Dox-ACF and the suppression of Bcl-2 expression on MCF7 cells. Nevertheless, the exact mechanism remains unknown and needs further investigation.

ACKNOWLEDGEMENT

This work was supported by Hibah Bersaing XV, Direktorat Jenderal Pendidikan Tinggi (DIKTI).

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