

## Synergistic Effect of Arecoline in Combination with Doxorubicin on HeLa Cervical Cancer Cells

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### Abstract

Arecoline, the main alkaloid of *Areca catechu* L has been proven to possess cytotoxic activity against various cancer cell lines. The research conducted to examine the cytotoxic activity of arecoline alone and its combination with doxorubicin against HeLa cervical cancer cell line. Single treatment of arecoline in various concentration on HeLa cancer cell were done followed by the combinational treatment with doxorubicin. The cell viability as the parameter of cytotoxicity was measured using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The apoptotic effect was examined by double staining assay using etidium bromide-acridin orange. Arecoline did not show potent cytotoxicity effect against HeLa since the value of  $IC_{50}$  is 462  $\mu$ M. The combinational treatment of arecoline and doxorubicin showed synergicity with the optimum CI value is 0.48 given by the treatment of 30mM arecoline combined with 125 nM doxorubicin. The result of this study shows that arecoline has potential to be proposed as co-chemotherapeutic agent for cervical cancer. However, further study on its molecular mechanism needs to be conducted.

**Keywords:** cinnamon essential oil, doxorubicin, T47D cells, combination cytotoxicity

### INTRODUCTION

Cervical cancer is the second most common cancer found in women all over the world. International Agency for Research in Cancer reported that in 2008 there are 532.232 cases of cervical cancer in 100 million women. One of chemotherapeutic agents that commonly used is doxorubicin. Unfortunately, a recent study showed that high dose of doxorubicin may cause cardiotoxicity in human (Gunawan, 2007). It is possible to lower the dose of doxorubicin for therapy but it will also lessen the activity (Wattanapitayakul, *et al.*, 2005). Therefore, it is needed to develop a new approach of treatment such as using co-chemotherapeutic agents.

Arecoline ( $C_8H_{13}NO_2$ ) is an alkaloid compound found in pinang fruit (*Areca catechu* L.). Some *in vitro* study showed that arecoline have cytotoxicity towards oral and liver cancer cells, and induce apoptosis in basal cancer cells (Chang, *et al.*, 2001; Cheng, *et al.*, 2010). Arecoline is a promising compound to be used as co-chemotherapeutic agent of doxorubicin. Previous study showed that arecoline is able to induce cell cycle arrest in KB cell line (Chang,

*et al.*, 2001). It is recently reported by Cheng, *et al* in 2010 that arecoline may induce apoptosis in human hepatoma HA22T/VGH cell line through the by suppressing the production of Bcl-XL and Bcl-2 protein, increasing p53 production, and inducing the release of cytochrom c from mitochondrial membrane.

HeLa cells are known to express 2 kinds of oncogenes, E6 and E7. E6 binds with p53 fosforilated protein meanwhile the E7 binds with pRb protein (Jansen, *et al.*, 2006). The bonding of p53 protein by the E6 causing the increase of protein's degradation rate. Arecoline reported to have ability in increasing the p53 expression and the expression of the gen that encode the p21<sup>WAF1</sup> (Chou, *et al.*, 2009). Besides, the arecoline can also induce the expression of Bax, the proapoptotic protein and inhibits the Bcl-2 which is antiapoptosis protein on hepatoma cells HA22T/VGH (Cheng, *et al.*, 2010).

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## MATERIALS AND METHODS

### Tested Compounds

The main compounds used in this study was Arecoline Hydrobromide (VETRANAL<sup>®</sup> Sigma Aldrich Number of Catalog 46063) obtained from Warista Yogyakarta. The chemotherapy agent doxorubicin in the form of injection solution in the concentration of 50 mg/25 mL (Kalbe Farma) were obtained from Dr. Sardjito Hospital Yogyakarta.

### Cell Culture

HeLa cells were the collection of Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada. Cells were cultured in the DMEM culture media 1640 (Gibco) containing 10 % v/v *Fetal Bovine Serum* (FBS) (Gibco) and penisillin-streptomycin 100 µg/mL (Gibco) and incubated in CO<sub>2</sub> incubator (Herceus) in the 37°C themperature and 5% CO<sub>2</sub>.

### Cytotoxic Assay

The cytotoxicity assay were done by MTT assay. HeLa cells were distributed in to 96 well plates (Nunc) with 5000 in cells each well. The cells were incubated for 24 hours in the CO<sub>2</sub> incubator (Herceus) with arecolin both for the single and combinational cytotoxic assay. DMSO was used as the solvent. In the end of the incubation time, each well were added by 100 µL MTT reagent (Sigma) in the DMEM culture media (Gibco). The plate were incubated for 4 hours in 37°C themperature until the formazan crystal were formed (to observed the formazan crystal were done under the inverted microscope (Olympus CH-2)). After 4 hours of incubation the reaction were stopped by adding the SDS 10% as stopper reagent as many as 100 µL in each well and was incubated for 24 hours in the room themperature.

This proccess provide the color change in the cells' media. The yellow colour would turn into purple because of the dehydrogenase enzim activity in the living cells which breaks the tetrazolium rings from MTT reagents in to formazan crystal. The intensity if absorbance of the formazan crystal were obtained by the ELISA reader Bio-Rad microplate reader Benchmark serial no. 11565, Japan) with 595 nm wavelenght.

### Apoptosis Induction

The confluent culture of HeLa cells wre harvested and distributed in the concentration of  $5 \times 10^4$  cells/well on the coverslips (Nunc) and added by 1000 µL of culture media. Cells were incubated for 24 hours in the CO<sub>2</sub> incubator so that cells were adapted and in normal condition. Cells were given the treatment as control cells,  $1/16$  IC<sub>50</sub> arecoline,  $3/8$  IC<sub>50</sub> arecoline,  $1/8$  IC<sub>50</sub> doxorubicin, combination of  $1/16$  IC<sub>50</sub> arecoline with  $1/8$  IC<sub>50</sub> doxorubicin, and combination of  $3/8$  IC<sub>50</sub> arecoline with  $1/8$  IC<sub>50</sub> doxorubicin, and then incubated for 24 hours. In the end of incubation, the DMEM culture media were washed by PBS and the cover slips were lifted and placed on the object glass and were dropped by etidium bromide-acridine orange as many as 10 µL. The observation of cells morphology were done under the fluorescence microscope (Zeiss MC 80).

### Data Analysis

#### Cytotoxicity Assay

The absorbation of each well were converted in to the percent of cell viability. The percent of cell viability were calculated by the formula:

$$\%cell\ viability = \frac{abs\ of\ drug\ treated\ sample}{abs\ of\ control} \times 100\%$$

Based on the regretion equation were counted the IC<sub>50</sub> concentration (the concentration causing death fo 50% of population) so that can be determined the cytotoxic potency (Doyle and Griffiths, 2000).

#### 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 same response as D1 and D2, respectively. CI value acquired will allow the evaluation of Arecoline's potency in combinatorial treatment with doxorubicin on HeLa cells. Interpretation was done based on the classification listed in Table 1.

**Table I. 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

#### Apoptosis Assay Analysis

The green fluorescence showed the living cells. The early stage of apoptosis will undergo the chromatin condensation and will be green in colour. Meanwhile the late apoptosis cells will be broken in to the small parts and become orange in colour (Renvoize, *et al.*, 1998).

## RESULTS AND DISCUSSION

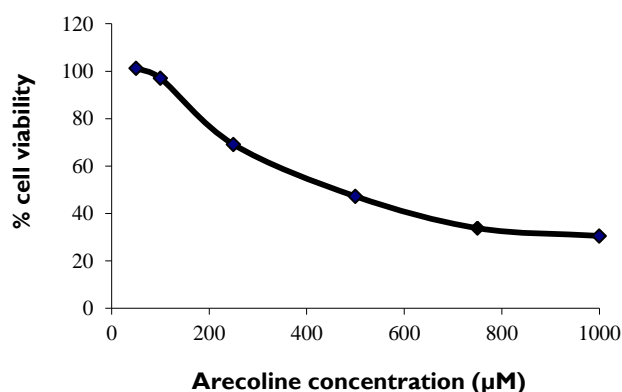
### Effect of Arecoline on HeLa Cells Growth

The cytotoxic assay was done to determine the cytotoxic potency of Arecoline as tested compound. The potency was expressed in the parameter of  $IC_{50}$ . The treatment of arecoline in the concentration of 1  $\mu$ M, 10  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 250  $\mu$ M, 500  $\mu$ M, 750  $\mu$ M, and 1000  $\mu$ M on HeLa cells caused HeLa cells growth decreased as the concentration of compound increased. Based on this study, the  $IC_{50}$  of Arecoline on HeLa cells is 462  $\mu$ g/mL (Fig. 1). The observation of HeLa cells morphology after the treatment of arecoline showed the cells death (cells become round in shape and deattached from the well plate)

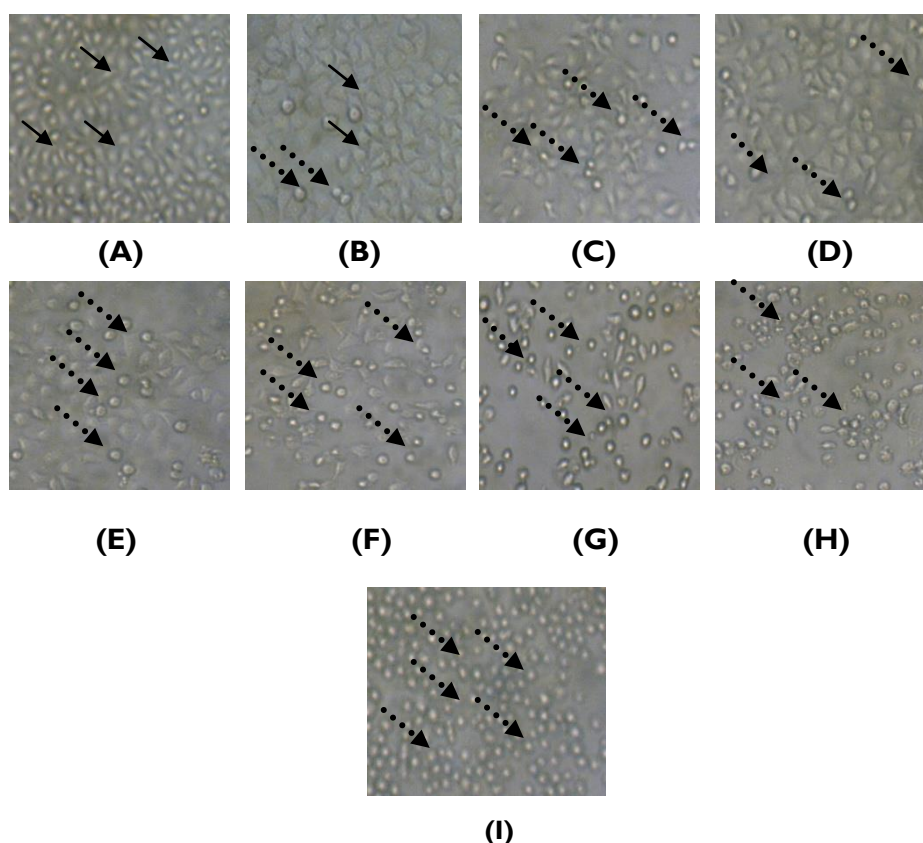
increased as the concentration of arecoline increased (Fig. 2)

This  $IC_{50}$  value of arecoline on HeLa cells showed that arecoline do not cause the strong cytotoxic activity on HeLa cells, compared with the other alkaloid such as scimmiamianin, flindercin, and haplopine (Jansen, *et al.*, 2006). Although the cytotoxic effect of this compound is not too strong, it is still possible for arecoline to be used as co-chemotherapy agent with doxorubicin.

Doxorubicin is known to have  $IC_{50}$  value 1000 nM on HeLa cells (Larasati, 2010). This showed that HeLa cells is not too sensitive with the treatment of doxorubicin. The  $IC_{50}$  value on HeLa cells is higher than the  $IC_{50}$  value of doxorubicin in other cancer cell such as T47D (43 nM) (Larasati, 2010; Jenie and Meiyanto, 2006). This could be caused by the resistance of doxorubicin on HeLa cells by the antiapoptotic protein in HeLa cells, ARC (*apoptosis repressor with caspase recruitment domain*) and efflux mechanism of doxorubicin from HeLa cells (Wang, *et al.*, 2009, Bravo-Cuellar, *et al.*, 2010). Therefore, the observation of combinational cytotoxicity of these two compound is needed to be done.



**Figure 1. The effect arecoline on the growth of HeLa cells.** As many as 5000 cells/well HeLa cells were incubated in the 96 well plates, and given the treatment of arecoline in the range concentration 1-1000  $\mu$ M.



**Figure 2. The effects of single treatment of arecoline on the HeLa cells morphology.** As many as 5000 cells/well were incubated in the 96 well plates. The observation of cell's morphology were done by using inverted microscope with 100x magnification. The figure showed control cells (A), cells treated by arecoline 1  $\mu$ M (B), 10  $\mu$ M (C), 50  $\mu$ M (D), 100  $\mu$ M (E), 250  $\mu$ M (F), 500  $\mu$ M (G), 750  $\mu$ M (H), and 1000  $\mu$ M (I). The arrow  $\longrightarrow$  showed viable cells, meanwhile  $\cdots\cdots\longrightarrow$  showed death cells.

#### Effect of Arecoline Combined with Doxorubicin on HeLa Cells Growth

Combination assay was done by combining  $1/16$   $IC_{50}$ ,  $1/8$   $IC_{50}$ ,  $1/4$   $IC_{50}$ ,  $3/8$   $IC_{50}$  of Arecoline with  $1/16$   $IC_{50}$ ,  $1/8$   $IC_{50}$ ,  $1/4$   $IC_{50}$ ,  $3/8$   $IC_{50}$  of doxorubicin. The cells viability were quantified by combination index (CI). Table II showed that the optimum IC value

were obtained by combining  $1/16$   $IC_{50}$  arecoline with  $1/8$   $IC_{50}$  doxorubicin with IC value 0.48, and the combination of  $3/8$   $IC_{50}$  arecoline with  $1/8$   $IC_{50}$  doxorubicin with CI value 0.52. The synergistic effect of doxorubicin in the combination with Arecoline probably through the apoptosis induction mechanism so that the observation of this mechanism should be done.

**Table 2. Combination Index combination of CEO and doxorubicin**

Arecoline ( $\mu$ g)	Doxorubicin (nM)			
	62.5	125	250	375
30	0.76	0.48	0.72	0.88
60	0.79	0.84	1.17	1.83
120	0.62	0.81	1.72	1.77
175	0.56	0.52	1.06	1.39

Treatment with combination of CEO 37.5 µg/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 µg/mL) and doxorubicin did not reduce cell viability significantly compound to the doxorubicin solely (Fig. 2).

### Effect of CEO and Doxorubicin on Apoptosis Induction

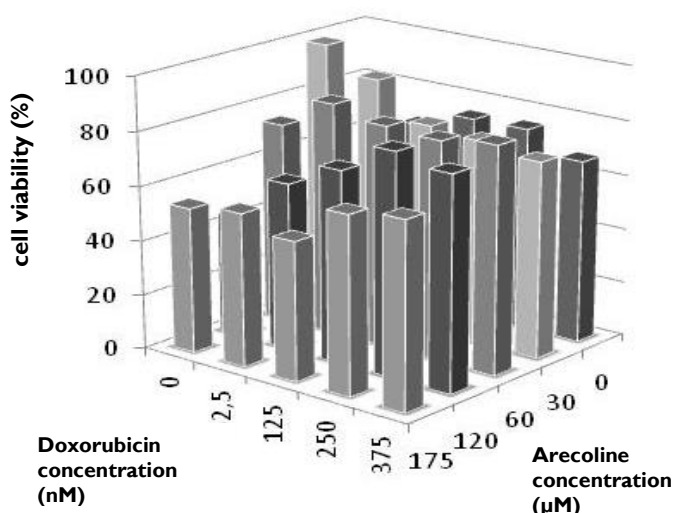
Double staining using ethidium bromide and acridin orange was used to observe the induction of apoptosis. Single treatment of CEO 37.5 µg/mL and doxorubicin 5 µM induced apoptosis of T47D cells. Apoptotic induction was indicated by orange fluorescent in the nucleus (Fig. 3). The result showed that combination 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.

The therapy of cervical cancer by doxorubicin needs high dose of treatment and usually cause the side effects such as nausea, hair loss, and cardiotoxicity. The synergistic effect of arecoline in the combination with

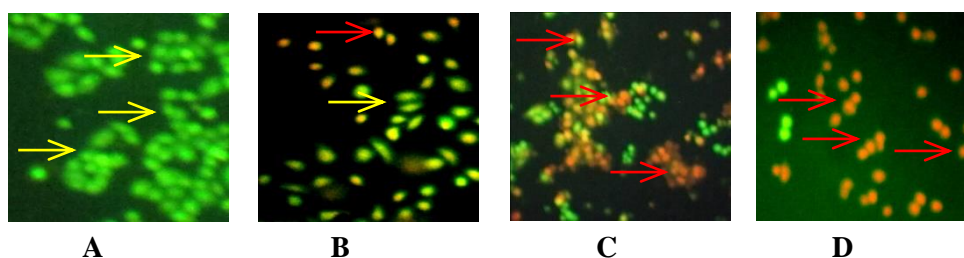
doxorubicin can reduce the dose of doxorubicin to obtain the same effect. As the dose is reduced, the side effect of doxorubicin can be reduced. Doxorubicin can induce the DNA strand break so that induce apoptosis. (Drummond, 2007). Arecoline also reported to induce the DNA strand break in mamalia cells (Chang, *et al.*, 2001). The synergistic effect of these two compounds are probably by the DNA strand break mechanism.

### The effect of arecoline and combination with doxorubicin in the apoptosis induction

The determination of the induction of apoptosis by the combination of Arecoline with doxorubicin in HeLa cells were done by apoptosis assay by double staining method. The result of this assay showed that the combination of arecoline and doxorubicin can induce the better apoptosis incidence than the single treatment of each compound. The apoptosis effect showed by the fluorescence of tge cells in to orang ecolour that caused by the decrease of membrane cells permeability. Some cells showed the nucleus fragmentation and the formation of apoptotic body (Fig. 4).



**Figure 3. The effects of combination treatment of arecoline with doxorubicin on the HeLa cells viability.** Cells were incubated for 24 hours and given the treatment of arecoline in combination with doxorubicin  $\frac{1}{16}$ IC<sub>50</sub>,  $\frac{1}{8}$  IC<sub>50</sub>,  $\frac{1}{4}$ IC<sub>50</sub> and  $\frac{3}{8}$  IC<sub>50</sub>. The graph shows HeLa cells viability after the treatment ( $p < 0.05$ ).



**Figure 4.** The effects of single and combination of arecoline and doxorubicin in the induction of apoptosis on HeLa cells. Control cells (A), Doxorubicin 125 nM (B), Arecoline 175 µM (C), and the combination of 175 µM arecoline and 125 nM doxorubicin. The yellow arrows → show the viable cells which green in fluorescence, and the red arrows → show the death cells which orange in fluorescence.

The induction of apoptosis by arecoline on HeLa cells probably by the p53 dependent mechanism and physical mechanism such as anoikis. This study showed the cytotoxic activity of arecoline and the synergistic effect of arecoline in the combination with doxorubicin on HeLa cells. The further research is need to be done to explain the molecular mechanism that mediate the cytotoxic activity of arecoline on HeLa cells and the synergistic effect with doxorubicin.

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