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Synergistic effects of *para*-hydroxy *meta*-methoxy chalcone (*p*H*m*MC)-doxorubicin treatments on T47D breast cancer cells

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Abstract

Resistance to some cancer chemotherapeutic drugs has been identified. One strategy to overcome that problem is by combining two or more of the drugs to get co-chemotherapeutic effects. A derivate chalcone, 3 - (4'-hydroxy-3'-methoxyphenyl)-1-phenyl-2-propene-1-on or *para* hydroxy *me*ta methoxy chalcone (*p*H*m*MC), has been reported to have cytotoxic activity on some cancer cells through some pathways. The aim of this study was to investigate the effects of combinations of *p*H*m*MC and Doxorubicin (Dox) on the cytotoxicity, anti-proliferation, apoptosis, and the cell cycle of T47D (breast cancer cell-lines) *in vitro*. The cytotoxic and antiproliferative activity were determined by MTT (3-[4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay. The combination index (CI) was used to determine the synergistic, additive or antagonistic effects of the combinations. Flowcytometry method was performed to determine the combination effects on the apoptosis and cell cycle. The results indicated that the combinations had a higher inhibitory effect on the cell growth compared to those of single treatments of *p*H*m*MC and Dox. All the doses used in the combinations were lower of the single doses at their IC_{50}s. The results showed all the combinations gave synergistic (CI: 0.3 – 0.7) up to strong synergistic (CI: 0.1 – 0.3) effects. The synergistic effects of the combinations were due to increased apoptosis and induced cell cycle arrest in S and G2/M phases on the cancer cell lines.

Keywords: *p*H*m*MC, Dox, co-chemotherapy, T47D cells.

Introduction

The American Cancer Society has reported that breast cancer is the second fatal cancer disease on women in the world (Anonymous, 2012). In Indonesia, breast cancer is the second most frequent one after cervix cancer and become the major caused on women mortality. The statistic data in 1991 reported that breast cancer cases reached 17.77% of all cancer cases in Indonesian women (Tjindarbumi, 2002). There are many cancer therapeutic strategies including surgery, chemotherapy, and radiation (King, 2000), but some problems found in the treatment. The main problems are the low selectivity and resistance of the drug on the tumor cells (Wong *et al*., 2006). Accordingly, any efforts in developing compounds which selectively against cancer are still needed.

Doxorubicin (Dox) is a cancer chemotherapeutic agent, which is given to various types of cancer, has been known to cause resistance. This compound includes in the anthracycline group. Besides resistance, it also causes cardiotoxicity in the long-term use (Ferreira *et al*., 2008). Co-chemotherapeutics approach is one of the alternative methods to resolve resistance problem. The method was combining the non-toxic or less toxic chemical with chemotherapeutic agents to

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enhance the efficacy with a reduced toxicity to normal tissue (Sharma *et al*., 2004; Tyagi *et al*., 2004).

Chalcones are α,β−unsaturated ketones which have the core structure of 1,3-difenilpropen-1-one. They have been studied as anti-cancer agents on different cell and animal models (Sasayama *et al*., 2007). One of the previous studies has reported that most actions of chalcones as cancer chemotherapeutic agents are blocking the cell cycle progress and inducing apoptosis (Hsu *et al*., 2006). Several kinds of literature also revealed that chalcones act on different types of targets (Boumendjel *et al*., 2009) including inhibitions of tubulin assembling (Ducki, 2009), angiogenesis (Mojzis *et al*., 2008), and kinases (Reddy *et a*l., 2010); as well as modulating of ABC proteins which involve in multidrug resistance (Valdameri *et al*., 2012), inductions of anti-estrogenic effect (Ducki, 2007) and non-apoptotic cell death (Robinson, *et al*., 2012).

Shen *et al*.(2007) have proved that the core structure of chalcones inhibits the activation of nuclear factor kappa (NF-κB), a transcription factor that is very important in the development and progression of cancer. It regulates many genes involved in inflammation, cell survival, cell proliferation, invasion, angiogenesis, and metastasis (Pahl, H.L., 1999). The inhibition of NF-κB activation causes apoptosis induction, cell cycle inhibition, and reduction of Bcl-XL expression as the downstream targets of NF-κB in T24 and HT-1376 bladder cancer cells, as well as MCF-7 and MDA -MB-231 breast cells (Hsu *et al*., 2006; Guttridge *et al*.,1999; Hinz *et al*., 1999).

Arty (2010) has been synthesizing some chalcone-derivative compounds with a hydroxyl group at the para position. One derivate resulted which has structure of (3- (4'-hydroxy-3'-methoxyphenyl)-1-phenyl-2-propene-1-one) named as para hydroxy meta methoxy chalcone (*p*H*m*MC) (Figure 1). The compound had cytotoxic activity against HeLa, Raji, T47D, and MCF-7 cancer cells. It was shown that it inhibited the proliferation of T47D and MCF-7 cancer cells by blocking the cell cycle progression. In MCF-7, *p*H*m*MC also induced the cell apoptosis (Arianingrum *et al*., 2012; Arianingrum *et al*., 2015). Interestingly, it did not have cytotoxic activity against Vero normal cell culture (Arianingrum *et al*., 2010). Based on these *p*H*m*MC's anti-cancer potencies, this study is to investigate the probable co-chemotherapeutic effect of *p*H*m*MC in combination with Dox.

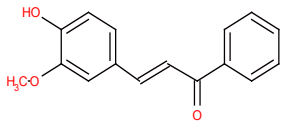

Figure 1. Structure of (3- (4'-hydroxy-3'-methoxyphenyl) -1-phenyl-2-propene-1-one) or para hydroxy meta methoxy chalcone (*p*H*m*MC)

Materials and Methods

Materials

The *p*H*m*MC was obtained from Prof Indyah Sulistyo Arty, Faculty of Mathematics and Natural Sciences, Universitas Negeri Yogyakarta, Indonesia. The compound was used as a stock solution with a concentration of 100 μM in dimethylsulfoxide (DMSO). The final concentration of DMSO in the study wells was kept less than 0.1%. Chemotherapeutic agent doxorubicin was from Kalbe, Indonesia.

Cell Cultures

T47D breast cancer cells were obtained from the collection of the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada, Indonesia. The cells were grown in a medium culture that contained RPMI 1640 (Gibco), 10% FBS (Gibco), 0.5% fungizone, and 1% penicillin-streptomycin (Gibco). The cells were developed at 37°C in a humidified atmosphere of 5% CO_2/95% air. Trypsin-EDTA 0.025% (Gibco) was used to detach the cells on the flask.

Cytotoxic and Anti-proliferation Assay

The cytotoxic and anti-proliferation tests were performed using MTT colorimetric assay. T47D cells were seeded at a density of 10^4 cells per well for the cytotoxic assay and $5x10^3$ cells per well for the anti-proliferative assay. The cells were grown for 24 hours in a humidified incubator at 37°C. After seeding, the cells were treated with solutions of *p*H*m*MC, Dox, and their combination. They were then incubated for 24 hours for the cytotoxic assay; and 0, 24, 48, and 72 hours for the antiproliferative assay. After incubations, the culture medium was removed and the cells were washed with 100 µL PBS (Sigma). The washed cells in the wells were added with 100 µL of MTT (Sigma) solution (0.5 mg/ml diluted with RPMI medium) and incubated for 4 hours at 37°C. The viable cells will react with MTT to produce purple formazan crystals. A 100 µL stopper reagent (10% SDS (Sigma) in 0.01M HCl) was added to dissolve the formazan crystal. The cells were then incubated for 12 hours (overnight) at room temperature and protected from light to determine the absorbance of the color formed due to the viable cells. The absorbance of each well was measured using ELISA reader (Bio-Rad) at λ 595 nm; and it was converted to a percentage of viable cells (Mosmann, 1983). The concentrations of *p*H*m*MC, Dox, and their combination at the IC_{50} were calculated. The co-chemotherapeutic effect of the combination solutions was analyzed using the Combination Index (CI) (Table 1) (Doyle and Griffith, 2000).

Table 1. Interpretation of Combination Index (CI) values

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

The CI was calculated based on equation below:

$$\mathbf{CI = D1/Dx1 + D2/Dx2}$$

D1 : combination concentration of *p*H*m*MC
D2 : combination concentration of Dox
Dx1 : concentration of *p*H*m*MC in single dose that could inhibit the T47D cells growth at the same point with combination concentration
Dx2 : concentration of Dox in single dose that could inhibit the T47D cells growth at the same point with combination concentration

(Reynold and Meurer, 2005)

The concentrations of *p*H*m*MC and Dox used in the combination were referred to the concentration of each single compound at its IC_{50}. The IC_{50} value of *p*H*m*MC in the previous study was 48 µM (Arianingrum *et al*., 2012); while the IC_{50} value of Dox was measured before the CI determination. To get a linear regression of Dox treatment versus viable T47D cells, concentrations of Dox of 7.81, 15.63, 31.25, 62.5, 125, and 250 nM were used for the treatment.

Analysis of the Apoptosis

T47D cells were seeded and put on a six tissue culture well-plate at 5×10^5 cells per well then incubated for 24 hours. The cells then were treated with various combination concentrations of *p*H*m*MC and Dox with each around its own IC_{50}. After 24 hours incubation, the cells were removed using 0.25% trypsin solution, then were centrifuged at 2000 rpm for 3 minutes and washed twice with cold PBS. The cells were re-suspended in 500 µl of Annexin V buffer (Roche), then were treated with Annexin V and propidium iodide (PI) for 10 minutes at room temperature and protected from light, then were analyzed using flow cytometer. The flow cytometer resulted four quadrants of the lower left (R1),

marked in blue, indicated viable cells; the lower right (R2), marked in green, indicated early apoptotic cells; the upper right (R3), marked in orange, indicated late apoptotic cells; and the upper left (R4), marked in yellow, indicated necrotic cells.

Analysis of the Cell Cycle

The washed T47D cells obtained from the combination treatment of *p*H*m*MC-Dox plate above were re-suspended in 500 µl of cycletest™ Plus DNA Reagent Kit (BD Biosciences). The Cellquest program, which works based on the DNA content, was used to calculate the percentage of the distribution of cells in each stage of the cell cycles (G1, S and G_2/M phases).

Results

Cytotoxic Effect of Doxorubicin

In this study, the IC_{50} of the *p*H*m*MC was got from the previous study of 48 µM (Arianingrum *et al.*, 2012); while the IC_{50} of the Dox was got from the Dox treatment versus viable T47D cells. The linear regression of Dox-cytotoxic effect resulted (Table 2 and Figure 2) gave the IC_{50} of Dox of 84 nM. Dox treatments have also caused cells morphology changing as presented in Figure 3. Some cells appear became rounded and detached from the bottom flask with a massive morphology. The rounded shape of cells indicated the mortality of T47D cells.

Table 2. The cytotoxic effect of Doxorubicin on T47D cells

Dox conc. treatment (nM)	Viable T47D cells (%)	Regression	IC_{50} (nM)
7.8	20,767	y=-52,308x + 150,69 R^2=0.9734	84
15.6	46,140		
31.3	57,854		
62.5	68,690		
125	94,489		
250	99,814		

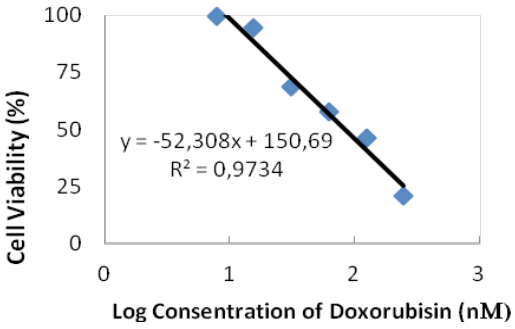

Figure 2. The linear regression of the Dox cytotoxic effect on the T47D cells viability.

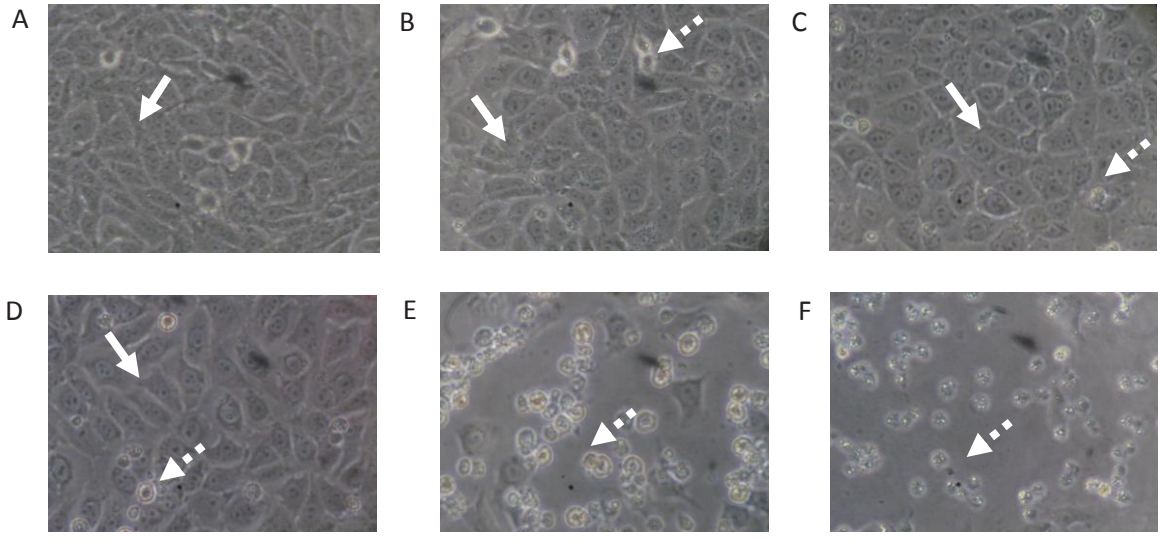

Figure 3. The T47D cells morphology: (A) with Dox-untreated, (B) treated with Dox at concentration of 7.8 nM, (C) 15.6 nM, (D) 31.3 nM, (E)125 nM, and (F)250 nM respectively. Observation of cell morphology was performed using inverted microscope with a magnification of 100x. The bold arrows indicate normal living cells, whereas the dashed arrows indicate the cell morphological changes.

The changes were more apparent in line with increasing concentrations of Dox.

Co-chemotherapeutic effect invitro of pHmMC-Doxorubicin on T47D cells

a. The cytotoxic effect of the *p*H*m*MC-Dox treatment

Several combinations of *p*H*m*MC-Dox were constructed with concentrations which were lower than the IC_{50} values of the single compounds. IC_{50} value of *p*H*m*MC from the previous study was 48 µM (Arianingrum *et al.*, 2012). The *p*H*m*MC concentrations used in treatment were 12; 18; 24; and 36 µM, while the Dox concentrations were 21; 31,5; 42; and 63 nM. The cytotoxic effect of the combinations of *p*H*m*MC and Dox were presented in Figure 4. The data showed that the combinations treatment of *p*H*m*MC-Dox more decreased the viability cells compared to that of the single treatments of *p*H*m*MC and Dox. Treatment of 18 µM *p*H*m*MC on T47D cells caused the cell viability of 60.97% and treatment of 31.5 nM Dox resulted in 67.08% cell viability. While the combination treatment at the same concentrations of the

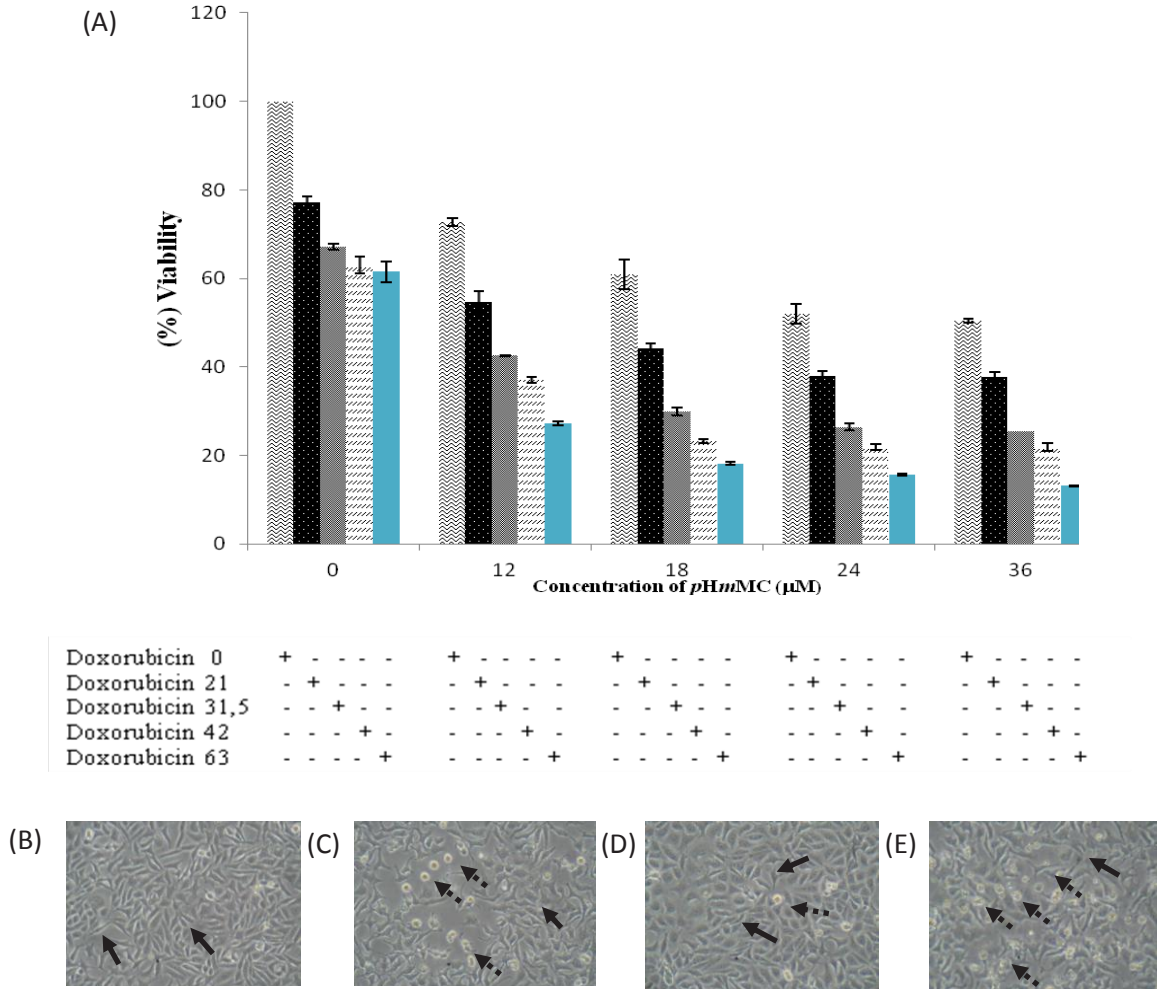

Figure 4. The cytotoxic effect of *p*H*m*MC-Dox combination on T47D cell. Graph (A) showed that the combination of *p*H*m*MC and Dox decreased cell viability compared to the single treatments. The combination treatment altered cell morphology: (B) untreated cells, (C) 18 µM *p*H*m*MC, (D) 31.5 nM Dox, and (E) combination of 18 µM *p*H*m*MC and 31.5 nM Dox. The bold arrows indicate normal living cells, whereas the dashed arrows indicate the cell morphological changes. The cell morphology was observed using an inverted microscope with 100x magnification.

single compounds resulted in cell viability was 29.93%. The higher concentrations of the single compounds in the combination gave decreasing cell viability. In this study, the lowest cell viability was obtained at combination concentrations of *p*H*m*MC and Dox of 36 µM and 63 nM respectively.

b. The combination index of the *p*H*m*MC-Dox treatment

The co-cytotoxic effects of the combination treatments were confirmed by the CIs resulted (Table 3). It was indicated that the combination treatments with concentrations of *p*H*m*MC and Dox above respectively 18 µM and 31.5 nM gave strong synergistic effect (CI=0.2). Combination treatments with lower concentrations of those showed weaker synergistic effects with CI values of 0.3, 0.4 and 0.7. The combination treatments also caused some morphological changes of the cells. The changes were more obvious in the combination treatment compared to the single treatments. Such changes included larger volume of the cells and cell nuclei, longer cell shape, more transparent and flat, rounded cell shape and cell floating. The study showed that the *p*H*m*MC increased the Dox-cytotoxic effect on the T47D cells.

Table 3. The combination index of the *p*H*m*MC-Dox treatments of on T47D cells

***p*H*m*MC (µM)**	**Dox (nM)**			
	21	**31.5**	**42**	**63**
12	0.7	0.4	0.4	0.3
18	0.4	0.2	0.2	0.2
24	0.3	0.2	0.2	0.2
36	0.4	0.2	0.2	0.2

c. The anti-proliferation effect of the *p*H*m*MC-Dox treatments

The Dox concentration used in the study was 21 nM, while the *p*H*m*MC concentrations were 18 and 24 µM. The observation results of the cell proliferative effect of the single and combination treatments are presented in Figure 5. The results showed that combination *p*H*m*MC-Dox treatments decreased the cell viability compared to that of the single treatments of *p*H*m*MC and Dox. The increase concentration of *p*H*m*MC or Dox in the combination treatment caused decreasing of the cell viability. All the treatments, both the single and the combination, decreased the cell viability until 48-hour incubation. After 48 hour incubation, the single treatments at lower concentration (18 µM *p*H*m*MC and 21 nM Dox 21) did not have antiproliferation effect so that the cells could proliferate and grow. While the single treatment with higher concentration (24 µM *p*H*m*MC) and the combination treatments still have an antiproliferative effect which inhibited the growth of the cells until 72-hour incubation.

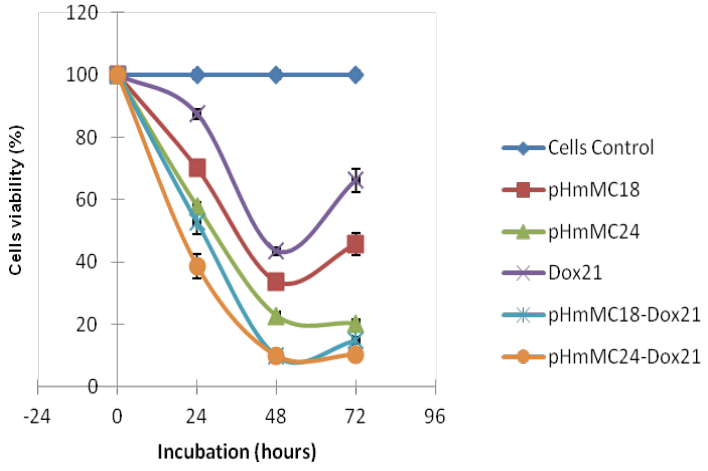

Figure 5. The inhibiting effects of *p*H*m*MC, Dox, and *p*H*m*MC-Doc treatments on the T47D cell proliferation.

The pHmMC –Dox Combination Effect on Apoptosis

The concentrations of *p*H*m*MC and Dox used for the apoptotic effect analysis were half of the IC_{50} which were respectively 24 µM and 42 nM. The results showed that all the treatments induced apoptosis on T47D cells at early and late phases as shown in Figure 6. After treatments with the *p*H*m*MC, Dox, and *p*H*m*MC-Dox, the percentage of late apoptotic cells increased by 12.05%; 2.21%; and 21.71% respectively from that of the control cells (2.05%). The percentage of the early apoptosis also increased by 3.94%; 2.31%, and 6.87 %

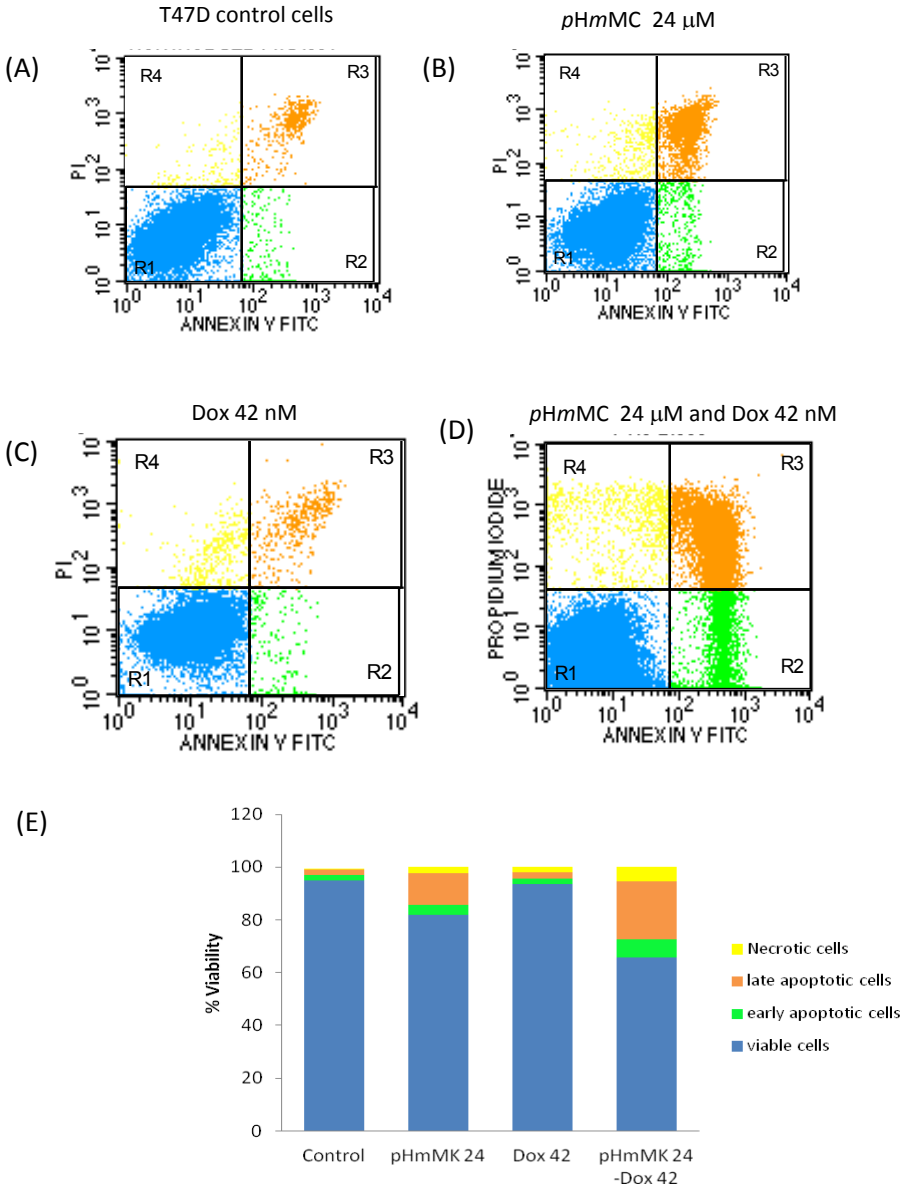

Figure 6. The effect of *p*H*m*MC, Dox, and their combination on T47D cells apoptosis. The flowcytometric profiles of (A), (B), (C), and (D) are the profiles of cell apoptosis after the treatments with respectively 0 µM (control), 24 µM *p*H*m*MC, 42 nM Dox, and the combination of 24 µM *p*H*m*MC-42 nM Dox. The Graph (E) shows the percentage of cell viability after the treatments. The longer the blue bar indicates the more viable cells; the longer the green and brown bars indicate more induce the cell apoptosis.

respectively from the control cells (2.09%). Compared with the single treatments, the combination treatments more induced the cell apoptosis.

pHmMC –Dox Combination Effect on cell cycle progression

The concentrations of the *p*H*m*MC and Dox used for analyzing the combination treatment effect on the T47D cell cycle were the same as the doses for the apoptosis study (24 µM *p*H*m*MC, 42 nM Dox, and their combination). Table 4 shows the distribution cells into cell cycle phases of Go-G1, S, and G2/M after the treatments. It shows that the single treatments of *p*H*m*MC and Dox inhibited the cell cycle and caused the cells arrest at G2/M, which were higher than that of the cell control. The *p*H*m*MC-Dox combination caused cells accumulation in S and G2/M phase.

Table 4. The inhibiting effects of *p*H*m*MC, Dox, and *p*H*m*MC-Doc treatments on the T47D cell cycle

Treatment	The Percentage of T47D cells (%) in phase :		
	G0-G1	S	G2/M
Untreated	59.96	22.51	16.72
24 µM *p*H*m*MC	47.88	18.41	29.08
42 nM Dox	25.99	24.98	47.61
24 µM *p*H*m*MC- 42 nM Dox	26.73	31.99	40.66

Discussion

From the study results, it was concluded that the *p*H*m*MC increased the Dox efficacy *in vitro* on T47D breast cancer cells. The CI study showed that the combination treatment of *p*H*m*MC-Dox had a synergistic effect up to strong synergy; especially at the low concentrations (below IC_{50}). The combination treatments also gave better anti-proliferative effect than that of the single ones. Those above facts confirmed that combining Dox with *p*H*m*MC could reduce the dosage of the Dox. This will give the benefit of minimizing the cytotoxic effects of the treatment to the normal cells.

The cytotoxic activity of the *p*H*m*MC, either as a single treatment or in combination with Dox, underwent the cell apoptosis and cell cycle arrest.

Studies on chalcones have been reported that apoptotic mechanism of the chalcones' treatments are occurred through mitochondrial pathway and inhibiting of NF-κB (Hsu *et al*., 2006; Shen *et al*., 2007; and Yadav *et al*., 2011); through increasing transcription of Bcl-2 that prevents the release of cytochrome c by Bax in mitochondria. The inhibition of NF-κB also causes decreasing of Bcl-2 and Bcl-XL expressions, two main anti-apoptotic proteins (Simstein *et al*., 2003). These mechanisms might be responsible for the apoptotic induction of *p*H*m*MC.

The single treatment of *p*H*m*MC induced cell arrest at G2/M phase. This result is consistent with the previous research (Arianingrum *et al*, 2012). While the Dox and *p*H*m*MC-Dox combination treatments induced cell arrest at S and G2/M phase. Various studies have shown that some chalcones inhibited cell cycle (Yadav *et al*., 2011). Some literature suggests that chalcones' ability to inhibit the cell cycle by inhibiting cell division cycle25B (Cdc25B) phosphatase (Zhang *et al*., 2014 and Yuan *et al*., 2014). Cdc25B is a mitotic regulator that acts as a 'starter phosphatase' to initiate the positive feedback loop at the entry into M phase. The activity of Cdc25B appears during late S phase and peaks during G2 phase. Both in-vitro and in vivo cdc25B is activated through phosphorylation during S-phase. Inhibition of phosphatase results in inhibition of cell cycle in G2/S-phase and mitotic arrest occurs (Lammer *et al*., 1998; Nilsson *et al*., 2000, and Lindgvist *et al*., 2005). While some study about Dox mention that Dox damaged double stranded DNA by intercalation on DNA base pairs and inhibition topoisomerase IIa. DNA damage will activate kinase protein (ATM), and this will further activate Chk2. Activation of Chk2 will cause Cdc25 inactive which in turn inhibit as cdc2. Both proteins are needed in G and M phase of cell cycle. Inactivation of these proteins will lead to G2/M arrest in the

cell cycle (Drummond, 2007). Combination treatment of *p*H*m*MC and Dox caused cell cycle arrest in S and G2/M. Combination treatment also decreased the cell population. Based on the research, *p*H*m*MC could improve Dox cytotoxic effect by increasing the inhibition of cell cycle arrest from G2-M to S phase.

Conclusion

The combination treatments of the *p*H*m*MC-Dox resulted in a synergistic up to strong synergistic effect, an increase in apoptosis, and induction of the cell cycle arrest in S and G2/M phase of T47D cancer cells. The combination treatment of *p*H*m*MC-Dox is potential to be developed as a breast cancer therapy.

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