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Pentagamaboronon-0 Fructose Inhibited Migration and Overexpression of Matrix Metalloproteinases 9 on MCF-7/HER2 Breast Cancer Cells

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Abstract

The incidence of Breast Cancer Metastasis (MBC) can be categorized in stage IV as well as being the leading cause of death in cases of breast cancer. MBC prognosis is known to be weak, frequent recurrent, and MBC patients have only a survival rate of about 5 years. One of the proteins that causes breast cancer metastasis is Human Epidermal Growth Factor 2 (HER2). Pentagamaboron-0 (PGB-0), a newly curcumin analogue performed cytotoxic effect on HER2-positive breast cancer cells but it is practically water-insoluble. The aims of this study are to determine anti-metastatic activity of a more soluble form of PGB-0 namely PGB-0 fructose complex (PGB-0-F) toward HER2 positive cancer (MCF-7/HER2) cells. PGB-0-F was obtained from Cancer Chemoprevention Research Centre Faculty of Pharmacy Universitas Gadjah Mada. Based on scratch wound healing assay result, PGB-0-F inhibited cell migration especially in combination with doxorubicin at the concentration 15 μM. Under gelatin zymography assay, PGB-0-F in combination with doxorubicin decreased Matrix Metalloproteinases 9 (MMP-9) expression compare to the doxorubicin. Hence, PGB-0-F has a potency to be developed as anti-metastatic agent on HER2 overexpression breast cancer.

Keywords : *HER2, MCF-7/HER2, PGB-0-F, Metastasis*

INTRODUCTION

Breast cancer is one of the most common types of cancer suffered by women (Ferlay, *et al*., 2012). Breast cancer was first as the most common cancer of women and the second leading cause of death due to cancer in women (Shiegel, *et al*., 2018). In Indonesia breast cancer ranks first with a percentage of 43.3% as a cancer that affects many women and causes at least 12.9% of deaths in women due to cancer (Kemenkes RI, 2015).

Breast cancer in the presence of excessive expression of Human Epidermal Growth Factor 2 (HER2) receptors is known to have a fairly high prevalence. It was reported that about 21% of 272 cases of breast cancer in 2006-2007 in Hong Kong

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were breast cancer with overexpression of HER2 (Yau, *et al*., 2008). In addition, 44.5% of breast cancer over HER2 expression was also found from 3,033 breast cancer patients in Iran (Keyhani, *et al*., 2010). Over-expression of HER2 in breast cancer can affect malignancy rates, affect metastasis and lead to uncontrolled growth of cancer cells (Gibbs, 2000; Lemmen, *et al*., 2004; Freudenberg, *et al*., 2009).

Metastasis can be interpreted as a process of transfer of cancer cells from the primary to other organs located far from the primary place (Scully, *et al*., 2012). Incidence of Breast Cancer Metastasis (MBC) can be categorized in stage IV as well as being the leading cause of death in cases of breast cancer. About 5% of patients diagnosed with breast cancer are MBC. MBC prognosis is known to be weak, recurrent is common, and MBC patients have only a survival rate of about 5 years (Donovan, 2013). In addition, therapy on MBC is purely palliative, *i.e.,* reducing symptoms, improving quality of life, lengthening survival time, and preventing cancer progression (O'Shaughnessy, 2005).

The treatment of breast cancer that is still in use today is based on the presence of excessive expression in protein markers such as estrogen receptor (ER), progesterone receptor (PgR) and also HER2 (Furuya, *et al*., 2012). Systemic treatments include the use of chemotherapy agents, hormonal agents and immunotherapy agents. The use of these three agents will initially give positive results in 90% of cases of breast cancer and 50% in cases of metastasis (Gonzalez-Angulo, 2013). However, intensive use leads to cancer resistance and new treatments are needed to address this problem.

Curcumin is a major component of turmeric that has been known to have strong cytotoxic activity so widely used as a lead compound for the discovery of new drugs (Figure 1). Some curcumin analogues have been previously known to have activities that are either anticancer agents or co-chemotherapy. Pentagamavunon-1 (PGV-1) had cytotoxic activity in WiDr cancer cells through apoptosis pathways (Meiyanto, *et al*., 2016) and was able to reduced the expression of angiogenesis factors (VEGF and COX-2) in estrogen-induced T474 cancer cells (Meiyanto, *et al*., 2006) Pentagamavunon-0 (PGV-0) also has antiproliferation activities in HeLa cells (Meiyanto, *et al*., 2003), T47D breast cancer cells (Meiyanto, *et al*., 2006) through induction of apoptosis from the caspase-3 pathway (Meiyanto, *et al*., 2007). Modification of PGV-0 with Potassium (K-PGV-0) also results in apoptosis and cell cycle induction, and antimigration of triple negative 4T1 breast cancer cells in combination with doxorubicin (Herwandhani, *et al*., 2016)

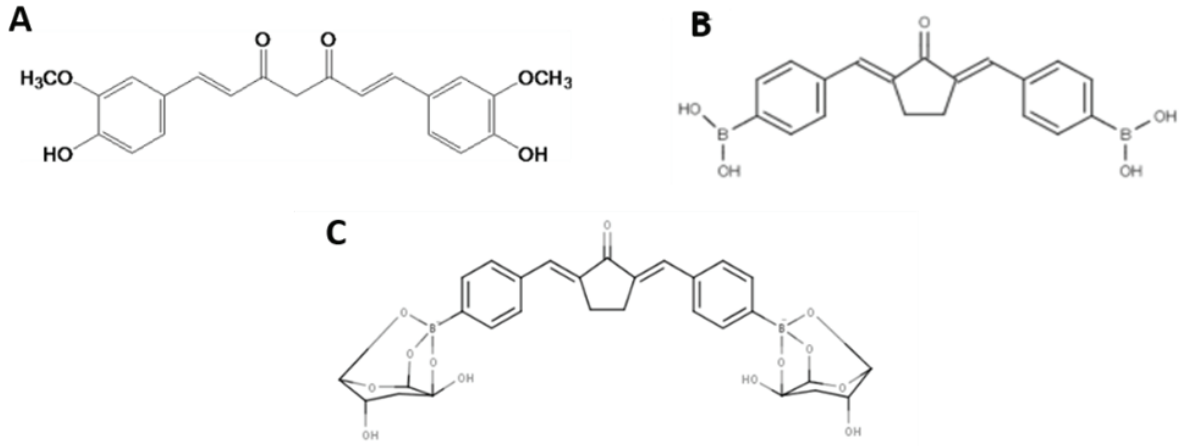

Figure 1. Chemical Structure of Curcumin (A), PGB-0 (B) and PGB-0-F (C).

One of the new curcumin analogues developed by Cancer Chemoprevention Research Centre Faculty of Pharmacy Universitas Gadjah Mada (CCRC-FF-UGM) is Pentagammaboron-0 (PGB-0). In addition to being a chemotherapy agent, PGB-0 also acts as a boron carrying agent that can be used as targeted chemotherapy. PGB-0 is known to have cytotoxic activity in some cancer cells but has a water-insoluble nature. Recent study reveals that PGB-0 have cytotoxic activity on MCF-7/HER2 breast cancer cells by modulated cell cycle in G1 phase. This activity is thought to be caused by binding of PGB-0 in the tyrosine kinase part of HER2 as evidenced by a decrease in HER2 expression in PGB-0 treatment (Utomo, *et al*., 2017). PGB-0 in combination with doxorubicin also showed antimigration by decreased the MMP-9 expression on 4T1 cells (CCRC unpublised data). Recent research has succeeded in making a complex between PGB-0 with fructose (PGB-0-F) which increases solubility of PGB-0. Increased solubility may increase uptake so as to also increase cytotoxic activity of PGB-0.

This study is intended to examine the solute complex capability of PGB-0 (PGB-0-F) as novel anti-metastasis agents on MCF-7/HER2 breast cancer cells. MCF-7/HER2 cells was used as a model of HER2 overexpression of breast cancer cells. Possible antimetastasis effect of PGB-0 was measured by scratch wound healing assay and gelatin zymograph. The result of this study will be used for further experiment in order to develop novel anti-metastasis agents from PGB-0-F.

MATERIALS AND METHODS

Materials

PGB-0-F was obtained from CCRC-FF-UGM. Doxorubicin was purchased from sigma.

Cell Culture

MCF-7/HER2 breast cancer cells stored in CCRC-FF-UGM was obtained from Prof. Kawaichi, Nara Institute of Science and Technology, Japan .The culture of MCF-7/HER2 cells was grown on a high glucose culture medium Dulbecco's Modified Eagle Media (DMEM) (Gibco, Invitrogen, Massachusetts USA) containing 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, Missouri, USA), 1.5% penicillin-streptomycin (Gibco) and 0.5% Fungizone (Gibco). Cells harvesting from Tissue Culture Dish (Iwaki, Kuala Lumpur, Malaysia) using 0.25% trypsin-EDTA (Gibco).

Scratch Wound Healing Assay

A total of 8.5 x 10^4 MCF-7/HER2 cells were distributed into 24 well plates, then incubated for 24 hours at 37°C, 5% CO_2 Cells were washed using PBS 1x and given starvation medium (a culture medium with 0.5% FBS) then incubated for 24 hours was. Each well then scratched vertically by using yellow tip and treated with PGB-0-F with concetration of 15, 7.5, and 3.75 μM and 10 nM Doxorubicin and their combinations. Cells were observed under a light microscope each time 0, 18, 24, and 42 hours and captured by digital camera (Samsung, Seoul, Korea).

Gelatin Zymography Assay

A total of 2 x 10^5 MCF-7/HER2 cells were planted in a 6-well plate with 2 mL of culture medium and incubated for 24 hours. The test solution was carried out using culture medium containing 0.5% FBS. After incubation, the media is removed and washed with PBS 1 mL 1 times. Cell then treated with various concentration of PGB-0-F and doxorubicin for 24 and 48 hours. Estradiol was used as an induction of Matrix Metalloproteinases 9 (MMP-9) expression. The 8% of SDS-PAGE supplemented with 0.1% of gelatin was used to determined the activity of MMP-9 in the culture medium. After electrophoresis, gels were washed and incubated with distilled water containing 2% of Triton-X 100 (Merck, Darmstadt, Germany) for 30 minutes at room temperature. The solution was removed from gels. 100 mL of reaction buffer (40 mM Tris-HCl pH 8, 10 mM $CaCl_2$, 0.02% NaN_3) was added and incubated for 24 hours at 37°C.

After removal of reaction buffer, gels were stained by Coomassie Brilliant Blue R-250 solution and destained by destaining solution (20% methanol, 10% acetic acid and 70% water) until clear bands with dark blue background appear. The results were documented and analyzed by ImageJ software.

Data Analysis

Scratch analysis was figured out by quantifying the cell migration distance using ImageJ software by comparing the distance between untreated and treated cells. Data from multiple scratch within the same test group were analyzed using Analysis of Variance (ANOVA) test to analyze the difference between experimental group. Data were presented as mean±S.D. The $p<0.05$ was considered statistically significant. Gelatin Zymography data analysis was performed by comparing the intensity of the band treated cells to untreated cells using ImageJ software.

RESULTS

Anti-Migratory Effect of PGB-0-F on MCF-7/HER2 Cells

Examination After 42 hours of incubation, single PGB-0-F treatment with 15 μM concentration gave the smallest closure value (60%) when compared with the lower PBG-0-F treatment of 7.5 μM (90%) and 3.75 μM (70%) (Fig. 2A). These results suggest that high concentrations of PGB-0-F may inhibit migration, but at low concentration will lead to an increase in MCF-7/HER2 cell migration .

Single doxorubicin treatment showed migration induction, whereas a single PGB-0-F treatment of 3.75 μM and 15 μM concentrations showed a decrease in cell migration when compared with control. This suggests inhibition of migration by PGB-0-F depending on the concentration (Fig. 2B). In the combination of PGB-0-F and doxorubicin seen that the combination of both can inhibit cell migration. When compared with a single treatment of doxorubicin, PGB-0-F appears to inhibit cell migration caused by the treatment of doxorubicin in MCF-7 / HER2 breast cancer cells.

Effect of PGB-0-F on MMP-9 Expression

The test results showed that MMP-9 expression significantly decreased by a single PGB-0-F treatment of 15 μM dosage either at 24 hours or 48 hours (Fig. 3). This decrease in MMP-9 expression is even smaller when compared to cell control. In the treatment of PBG-0-F low concentrations of 3.75 and 7.5 μM at 24 hours and 48 hours gave opposite results. This can be seen from the band's intensity which is thinner when compared to cell control. This indicates that MMP-9 expression is elevated in the low concentration PGB-0-F treatment but the increase in activity is still below that of a single doxorubicin treatment.

MCF-7/HER2 cells were given estradiol treatment to increase their metastasis ability, then be treated with doxorubicin and PGB-0-F. At 24 hours and 48 hours the combination of PGB-0-F 3.75 μM with doxorubicin led to an increase in MMP-9 expression and higher than a single doxorubicin treatment. However, the combination of PGB-0-F with doxorubicin at higher concentration resulted in decreased MMP-9 activity. Based on the results obtained, the possible combination of PGB-0-F with doxorubicin could decrease the expression of MMP-9 induced by doxorubicin.

DISCUSSION

In this present study, we observed the inhibition of cancer cell migration as the one of parts of metastasis process by treatment of PGB-0-F through scratch wound healing assay. Based on the percent graph of MCF-7/HER2 cell closure (Figure 2A) PGB-0-F single treatment showed a decrease in cell movement at concentrations of 3.75 μM and 15 μM. The treatment of PGB-0-F with concentration 15 μM has demonstrated highest migration inhibitory with % closure only 60% compare to the all treatment.

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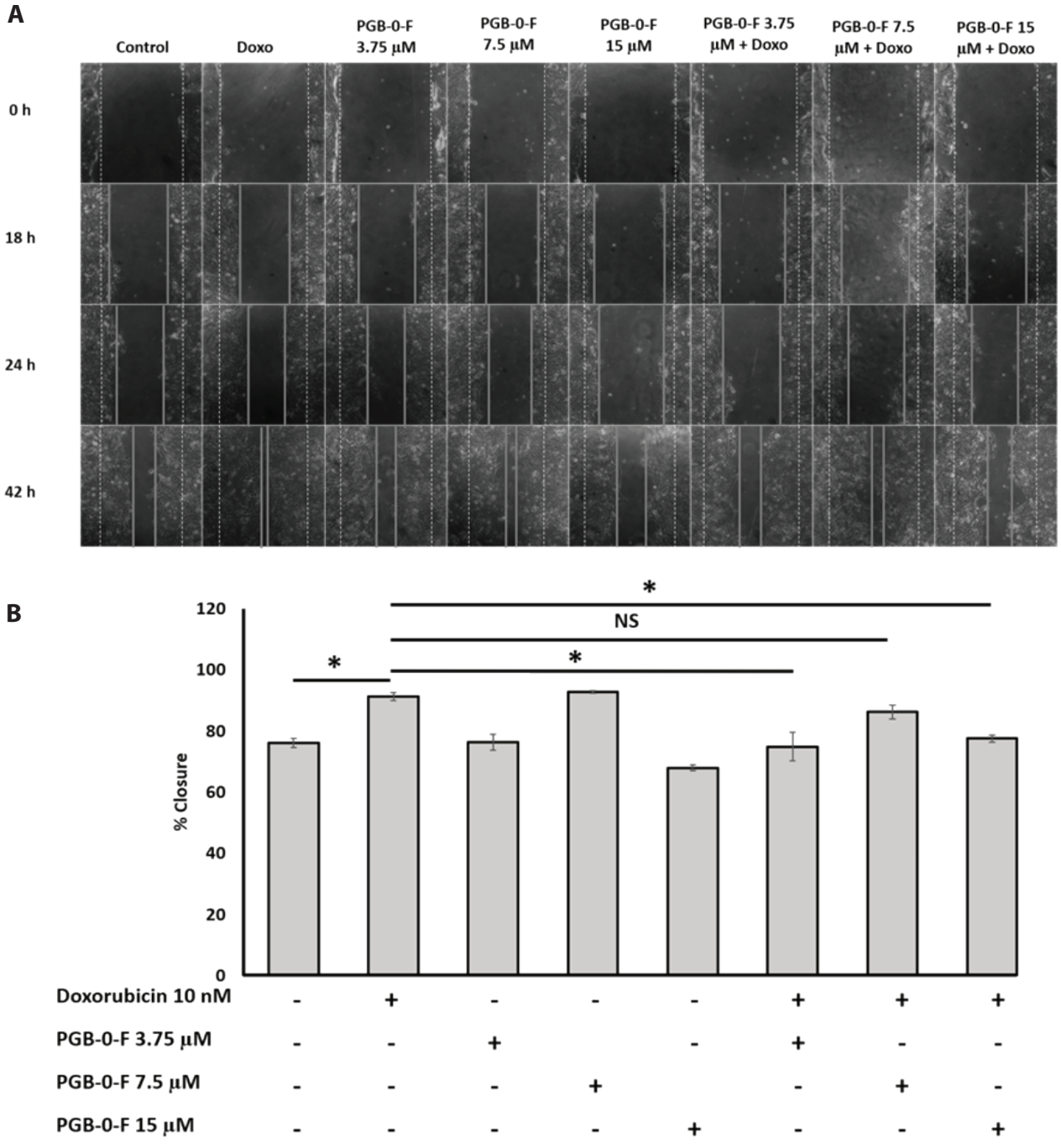

Figure 2. Effect of Single Treatment of PGB-0-F and its Combination with Doxorubicin on MCF-7/HER2 Cells Migration. A: The morphology of the cells after scratch and treated with 10 nM doxorubicin, 3.75; 7.5 and 15 µM PGB-0-F and their combination. Observations were made after 0, 18, 24 and 42 hours of treatment under an inverted microscope with magnification of 100x. B: The percentage of MCF-7/HER2 cells closure after 42 hours of treatment. The area of the scratch were analyzed using ImageJ software then % closure was calculated in accordance with the procedures of the analysis. *: significance different with $p<0.05$, NS: not significance different, N=3.

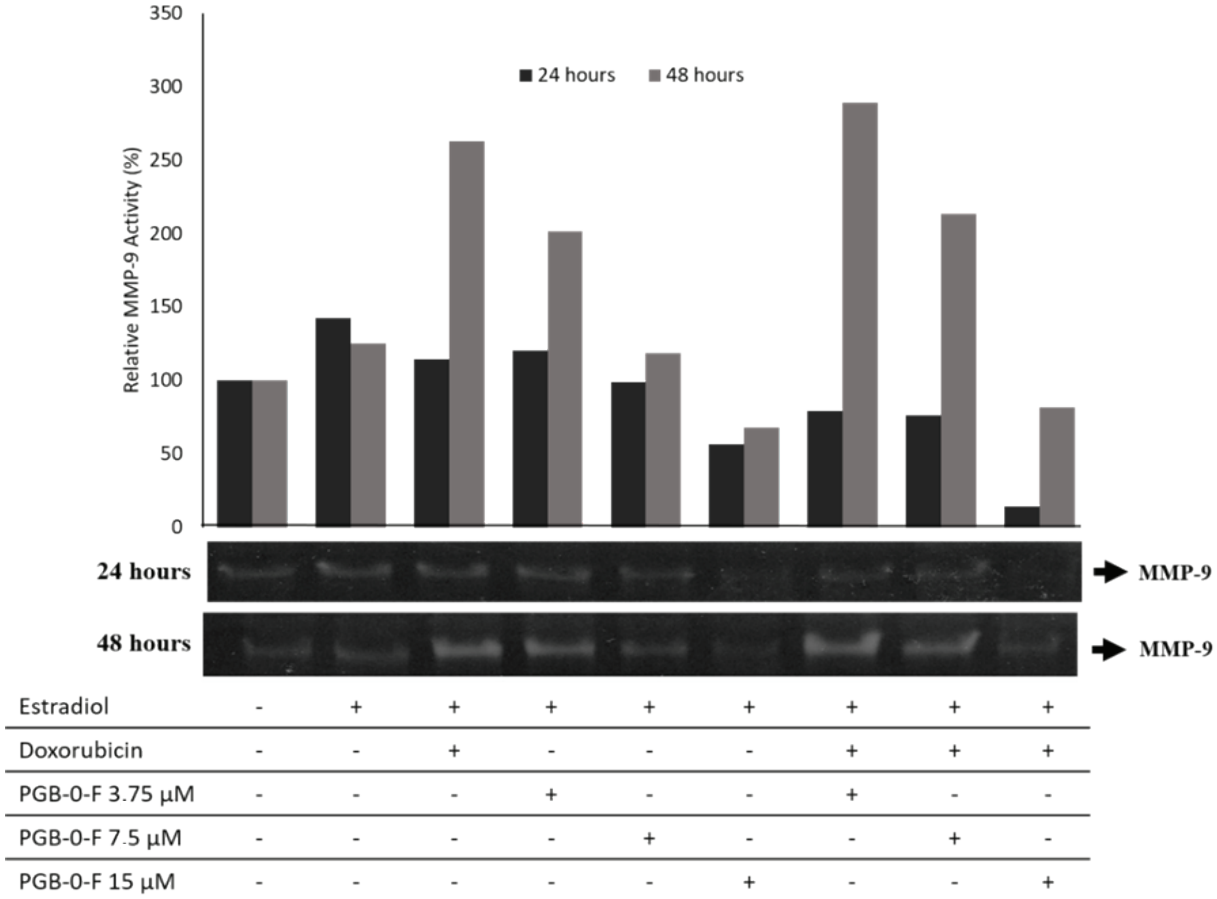

Figure 3. Effect of single treatment of PGB-0-F and its combination with Doxorubicin in MMP-9 activity on MCF-7/HER2 Cells. Cell were treated with 10 nM Doxorubicin, 3.75 µM, 7.5 µM, 15 µM PGB-0-F, and their combination for 24 and 48 hours. MMP-9 assay was conducted using gelatine zymography according to the method. Analysis of the result were done by using ImageJ software to measure the intensity of gelatin degradation by MMP-9 in the gel.

Doxorubicin is a chemotherapy that is widely used in cases of breast cancer. Doxorubicin occupies the main chemotherapy in the treatment of breast cancer, especially breast cancer with positive HER2 (American Cancer Society, 2006). In the treatment of doxorubicin acts both in the adjuvant or neoadjuvant parts along with other types of therapy. Recent study revealed doxorubicin at concentration of 10 nM is known to induce migration by induce the formation of lamelipodia but does not affect the proliferation of breast cancer cells 4T1 and MCF-7/HER2 (Amalina, *et al*., 2017). This result in this study on a significant increase of cell movement compared to control cells with % closure up to 90%. Furthermore the combination of PGB-0-F with doxorubicin gave inhibitory results of cell migration when compared with a single treatment of doxorubicin. This result shows PGB-0-F is able to suppress the induction of migration caused by doxorubicin and can act as co-chemotherapy agent along with doxorubicin. This result also in line with previous study which showed that curcumin and its analogues (PGV-0 and PGV-0) can increased the sensitivity of MCF-7 breast cancer cells that have resistance with doxorubicin through HER2 and NF-kB inhibition (Meiyanto, *et al*., 2014)

Normal cell migration is associated with excessive expression of extracellular matrix degrading proteins such as MMP-9. In the present study the MMP-9 expression was measured using gelatine zymography. The main principle of this method is to look at the ability of MMP-9 to degrade gelatin added in the gel and to reveal a clear band to the gel. MCF-7/HER2 cells were given estradiol treatment to improve their metastatic ability. The 17β-estradiol compound (E2) is an estrogen hormone known to increase the ability of movement and invasion of breast cancer cells by activating ezrin actin-binding protein (Zheng, *et al*., 2011). The treated E2 cells were then given a single PGB-0-F treatment, doxorubicin and a combination of both.

At a single treatment E2 hours 24 and 48 appear to increase MMP-9 expression as compared to cell control. In addition, an increase in MMP-9 expression was also seen in the treatment of doxorubicin especially at 48 hours. At a single 24-hour PGB-0-F treatment can decrease MMP-9 expression in line with increasing dosage. In combination PGB-0-F and doxorubicin the MMP-9 expression also decreased and looked like a combination of PGB-0-F with doxorubicin mutually impeding MMP-9 enhancement by both. Particularly in the treatment of PGB-0-F 15 μM concentration with doxorubicin decreased MMP-9 expression was lower when compared with cell control and single treatment of both compounds. While in the 48-hour treatment PGB-0-F single treatment also increased the expression of MMP-9 higher than the treatment of doxorubicin along with increased concentration. The combination of PGB-0-F treatment with doxorubicin also increased MMP-9 expression when compared with the second single treatment. These results show that in 48 hours treatment the combination of PGB-0-F concentrations of 3.75 μM, 7.5 μM and 15 μM with doxorubicin actually increased MMP-9 expression.

The migration test and MMP-9 expression were carried out at 24 hours and 48 hours because to see whether the treatment with PGB-0 could have a good effect on the condition before doubling time of MCF-7/HER2 cells or when the cell had proliferated. The increase in MMP-9 expression at 48 hours is thought to be due to doubling time and increasing number of cells, causing MMP-9 expression to increase. This can be seen with an increase in MMP-9 expression in each treatment. However, this increase can be overcome by a decrease in MMP-9 expression which is in line with the increase in concentration, especially in combination treatment. This is what explains if the PGB-0-F treatment combination with doxorubicin can reduce MMP-9 expression.

In the migration test, cell migration was increased in line with increasing concentrations in the PGB-0-F single treatment. Particularly for the treatment of PGB-0-F the concentration of 7.5 μM was seen to increase cell migration significantly when compared to cell control. Whereas in the combined treatment of migration results also increased when compared with control cells, but decreased when compared with single doxorubicin treatment. This suggests that the combination of PGB-0-F with doxorubicin gives a positive effect, *i.e*., each drug is able to negate the migration effect caused by the second single treatment. However, the MMP-9 test results show a decrease in MMP-9 expression in line with increasing concentrations in both single and combination treatments. This result is different from the results on the migration test. Decreased expression of MMP-9 indicates a decreased ability of cancer cell migration. However, in the present study migration persists even though MMP-9 expression decreases. It is possible that inhibition of migration by a combination of doxorubicin and PGB-0-F in MCF-7/HER2 cells is not through decreased MMP-9 expression, but through other pathways such as affecting the dynamics of actin cytoskeleton via RAC1 pathway (Welf and Haugh, 2011; Amalina, *et al*., 2016).

CONCLUSION

PGB-0-F exhibits anti-migration effect against doxorubicin treatment and also inhibits MMP-9 alone and combination with doxorubicin on MCF-7/HER2 cells.

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