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Pentagamavunon-0 (PGV-0) Enhance Cytotoxic Effect of Doxorubicin Through Increasing of Apoptosis, Senescence and ROS Level on Triple Negative Breast Cancer 4T1

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Abstract

Triple negative breast cancer (TNBC), one of the sub type of breast cancers was widely known with high tumorigenic and poor prognosis than others. The development of combination agent (co-chemotherapy) with doxorubicin for chemotherapy of TNBC were carried out to decrease doxorubicin side effect and resistance in cancer. This present study aims to explore the co-chemotherapeutic properties of Pentagamavunon-0 (PGV-0) and investigate induction of doxorubicin on apoptosis, senescence and Reactive Oxygen Species (ROS) against TNBC. 4T1 Cell line was used as a TNBC *in vitro* model. Cytotoxic measurement was performed using 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromide (MTT) assay resulting in IC_{50} values of 52 µM. Meanwhile, the combination of doxorubicin and PGV-0 showed synergistic effect which decreased cell viability of 4T1 better than single treatment of doxorubicin. Apoptosis analysis was performed using annexin V/PI assay indicated that the combination treatment of PGV-0 and doxorubicin increased apoptosis evidence. Senescence detection was carried out using senescence-associated-ß galactosidase (SA-ß-GAL) assay. The results showed that a single treatment of PGV-0 induced cellular senescence and increased senescence cells in combination treatment. Moreover, 2',7'-dichlorofluorescein diacetate (DCFDA) staining showed that PGV-0 increased ROS level at single treatment, whereas combination treatment increased ROS intracellular compared to the positive control of doxorubicin. Based on these results, PGV-0 has potential as a co-chemotherapeutic candidate on TNBC.

Keyword: *4T1, PGV-0, Co-chemotherapy, Cytotoxic, Senescence, Apoptosis, ROS*

INTRODUCTION

Triple negative breast cancer (TNBC), one of the highly tumorigenic of breast cancers is known as difficult to treat due to the lacking of estrogen receptor, progesterone receptor and human epidermal receptor-2 (HER-2) (O'Reilly, *et al.*, 2015). TNBC chemotherapy uses doxorubicin with a broad spectrum of action. Nevertheless, long term usage of doxorubicin can cause side effects such as cardiotoxicity and resistance of doxorubicin in cancer (Hanna, *et al.*, 2014). The

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recent development of TNBC chemotherapy directed to find a potential combination agent (co-chemotherapy) that can increase the effectiveness of doxorubicin. In this regard, doxorubicin is hopefully to forms a synergistic effect with co-chemotherapeutic agents which results in enhancing the cytotoxic effect of doxorubicin, thus decreasing the dose of doxorubicin to reduce the side effect and avoid the cancer cell resistance to doxorubicin.

Curcumin, a phenolic compound of turmeric (*Curcuma longa*) is known to have cytotoxic properties by targeting various proteins involved in cell survival, metastasis, and angiogenesis. A study reported that curcumin also increases Reactive Oxygen Species (ROS) level through interaction with ROS metabolism enzymes such as glutathione S-transferase and Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NAD(P)H) dehydrogenase (Larasati, *et al.,* 2018). The increase of ROS intracellular over cellular capacity tolerance of ROS could trigger senescence, a kind of cell cycle arrest that limits the replicative capacity of cells and induce apoptosis resulting in inhibition of the progression of cancer (Campisi, 2013). As co-chemotherapeutic agents, the combination of curcumin with doxorubicin enhances the cytotoxicity of doxorubicin in cancer through inhibition of efflux transporters which responsible for doxorubicin resistance (Tan and Norhaizan, 2019; Wen, *et al*., 2019). However, curcumin has low bioavailability that reduces the cytotoxic effect on *in vivo* study. Therefore, the development of curcumin analogs was carried out to find more potent compounds than curcumin.

Pentagamavunon-0 (PGV-0), one of the curcumin analogs was synthesized using vanillin and cyclopentanone. PGV-0 exhibits cytotoxic properties against several types of breast cancer such as T47D, Michigan Cancer Foundation-7 (MCF-7), and MCF-7/Dox (Da'i, *et al.*, 2012; Meiyanto, *et al*., 2014). The previous study reported that a combination of PGV-0 and doxorubicin showed an increase of cytotoxic and sensitivity effect of doxorubicin on MCF-7/Dox due to HER2 with PgP inhibition and activation in the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Meiyanto, *et al*., 2014). This present study aims to reveal the potential co-chemotherapeutic effect of PGV-0 on 4T1 cells, as a model of TNBC. Furthermore, this study also investigates its effect on apoptosis, cellular senescence, and ROS level. The result of this study can be used as a foundation of a further experiment for the development of PGV-0 for TNBC.

MATERIAL AND METHOD

Chemical Material

PGV-0 compound was obtained from Cancer Chemoprevention Research Center (CCRC), faculty of Pharmacy, Universitas Gadjah Mada. Doxorubicin was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Cell Culture

Cell line vero and cell line 4T1 (ATCCR-CRL-2539TM) were obtained from Prof. Masashi Kawaichi, MD., Ph.D (Nara Institute of Science and Technology, NAIST, Nara, Japan). Cell cultured on high glucose Dulbecco's Modified Eagles Medium (DMEM) (Gibco, New York, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, Missouri, USA) and 1.5% penicillin-streptomycin (Gibco) (v/v) (Gibco) and then incubated at 37°C with 5% CO_2. Cells were harvested from tissue culture disk (Iwaki, Tokyo, Japan) using 0.25% trypsin-Ethylenediaminetetraacetic acid (EDTA) (Gibco).

Cytotoxic Assay

Cytotoxic assay was performed by 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromide (MTT) cell viability assay (Mosmann, 1983). The 4T1 cell line ($2.65x10^3$ cell/well) and Vero cell ($1x10^4$ cell/well) were cultured at 96-well plate. The cells were treated with various concentrations of PGV-0 and incubated in CO_2 incubator for 24 h at 37°C. After 24 h, culture media

were removed and cells washed using Phosphate-buffered saline (PBS) IX. A total of 100 µL/well 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromide (MTT) solution (0.5 mg/mL) (Biovision, California, USA) were added. If formazan has been formed, a 10% Sodium Dodecyl Sulphate (SDS) solution (100 µL/well) was added then incubated overnight under room temperature. Absorbance measured using a microplate reader (BioRad, California, USA) at 595 nm. The value of IC_{50} was calculated using linearity regression of cell viability, while its value was used to analyze the selectivity index (SI) of PGV-0. The value of SI>2 indicated that PGV-0 selective on 4T1 (Koch, *et al*., 2005).

Combination Cytotoxic Assay

Cytotoxic combination assay was performed using modified of MTT cell viability assay that described on previous study of PGV-0 (Meiyanto, *et al*., 2014). The 4T1 cell line ($2.65x10^3$ cell/well) were cultured at 96-well plate and treated with combination of PGV-0 (12.5 µM, 25 µM, and 50 µM) and doxorubicin (10 nM and 100 nM) in culture medium (100 µL/well). After 24 h incubation on CO_2 incubator at 37°C, cells were washed using PBS IX and 100 µL MTT solution (0.5 mg/mL) (Biovision) was added each well. A total of 100 µL/well SDS solution (10%) added to stop the formazan forming reaction. The cells were incubated overnight under room temperature. Absorbance measured using a microplate reader (BioRad) at 595 nm. The calculation of combination index (CI) was carried out to analyze combination effect of doxorubicin and PGV-0. The value of CI used to determine combination effect of both compound, whether synergistic (CI<0.9), additive (CI:0.9-1) or antagonistic (CI >1.1) (Huang, *et al*., 2017).

Annexin V/PI Staining (Apoptosis Assay)

A total of 4T1 $1.5x10^6$ cells/well were cultured at 6-well plate. The cells were treated with PGV-0 at a concentration of 50 µM and combination with doxorubicin (10 nM and 100 nM). Treated cells were incubated for 24 h at 37°C and 5% CO_2. Cells were harvested using 0.25% EDTA and collected at microtube. The cells were fixed using cold ethanol (70%) for 30 minutes. After that, ethanol was removed from cells suspense, then added a solution of annexin V/PI (Roche, Hague Rd, Indianapolis, USA) 50µg/mL in binding buffer. Cells suspension was incubated in a CO_2 incubator for 10 minutes at 37°C. Apoptosis assay was performed using BD Accury C-6 Flowcytometer at 488 nm. Data were presented as a percentage of early apoptosis, late apoptosis, and necrosis (Lakshmanan and Batra, 2013).

Senescence-Associated β-Galactosidase (SA-β-GAL) Assay

The 4T1 ($1.5x10^6$ cell/well) were cultured at a 6-well plate. After 24 h incubation, medium culture was removed and the cells washed using 1X PBS. The cells were treated using PGV-0 singly (25µM and 50µM) and combination with doxorubicin at concentration 10 nM. Cells were incubated at CO_2 incubator for 24 h, 37°C. After that, the cells were fixed with paraformaldehyde (PFA) 4% for 10 minutes at room temperature, and then washed twice using 1X PBS. Each well given 1 mL of Senescence-Associated β-Galactosidase (SA-β-GAL) dye solutions and incubated in incubator non-CO_2 at 37°C. The cells were observed at 24, 48 and 72 h using an inverted microscope. The senescence cell was distinguished with blue-greenish color. Data were presented as the percentage of senescence cell (Eccles and Li, 2012).

Analysis of Intracellular Reactive Oxygen Species (ROS) Using 2',7'-dichlorofluorescein diacetate (DCFDA) Staining

The 4T1 cell line ($5x10^4$ cell/well) were cultured in culture media (DMEM) at 24-well plate. The cells harvested using 300 µL trypsin-EDTA. Trypsin was inactivated using 500 µL supplemented buffer and collected at microtube. A total of 25 µM DCFDA solutions were given each well. Cells were incubated in a CO_2 incubator, for 30

minutes at 37°C. After that, cells were treated with PGV-0 at concentration 50 µM and its combination with doxorubicin 10 nM. After 30 minutes of incubation, ROS analysis was performed using BD Accury C-6 Flowcytometer at 458 nm/535 nm. Mean fluorescence intensity was used to determine the ROS level on cells (Eruslanov and Kusmartsev, 2010).

RESULTS

Cytotoxic Effect of PGV-0

Several studies of PGV-0 revealed cytotoxic properties of PGV-0 on various types of breast cancer (Hermawan, *et al*., 2011; Meiyanto, *et al*., 2014). Nevertheless, the cytotoxic effect of PGV-0 on TNBC is not yet known. Therefore, we examine cytotoxic properties of PGV-0 on TNBC using a 4T1 cell line as a TNBC model and a Vero cell line as a normal cell. This research showed that PGV-0 has a cytotoxic effect on dose-dependent manner with IC_{50} values of 4T1 cells and vero cells were each 52 µM and 152 µM (Figure 1). The analysis of selectivity indicated that the cytotoxic effect of PGV-0 was selective on 4T1 cells (SI>2).

Cytotoxic Combination of PGV-0 with Doxorubicin

Based on the cytotoxic data above, PGV-0 showed great potential as an anticancer compound. We assumed that the inhibitory effect of PGV-0 was possibly enhancing the cytotoxic effect of doxorubicin on 4T1 cells. In this experiment, we investigate the cytotoxic effect of PGV-0 on 4T1 in combination with doxorubicin. We used PGV-0 at 12.5 µM, 25 µM, and 50 µM combined with doxorubicin at the concentration of 10 nM and 100 nM. The results of combination treatment (Figure 2) were reduced cell viability of 4T1 cells higher than single treatment of doxorubicin or PGV-0. The combination index value of each concentration less than 0.9 indicated a synergistic effect on 4T1 cells (Table 1). This data suggested that the cytotoxic effect of combination treatment is greater than the sum of each effect on a single treatment.

Apoptosis Induction Effect of Combination Doxorubicin and PGV-0

The combination of PGV-0 and doxorubicin resulted in improved cytotoxicity of doxorubicin on 4T1 cells. To confirm the cellular mechanism of these combinatorial treatments, we performed apoptosis analysis using annexin V/PI assay. The results show that PGV-0 at concentration 50 µM induces apoptosis on 4T1 cells, while combination treatment increase apoptosis effect was better than single treatment of PGV-0 or doxorubicin (Figure 3 and Table 2). However, looking at the data of

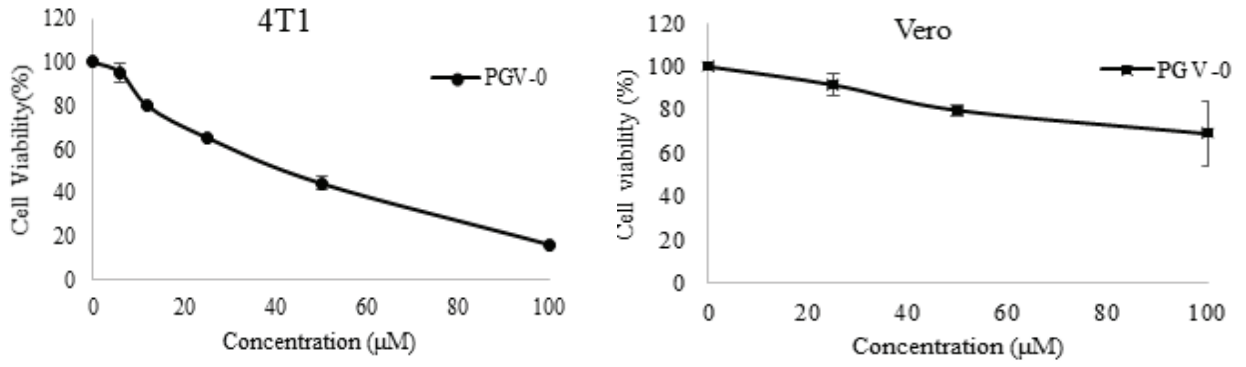

Figure 1. Cytotoxicity of PGV-0 on 4T1 and Vero cells. The 4T1 cells ($2.6x10^3$ cell/well) and Vero ($1x10^4$ cell/well) were cultured at 96 well-plate, then treated with various concentration of PGV-0. After 24 h incubation, MTT solutions were added. SDS were given to stop formazan forming reaction, then incubated overnight. The IC_{50} values was calculated from the triplicate experiments (A) cytotoxic effect of PGV-0 on 4T1, (B) cytotoxic effect of PGV-0 on Vero cell line.

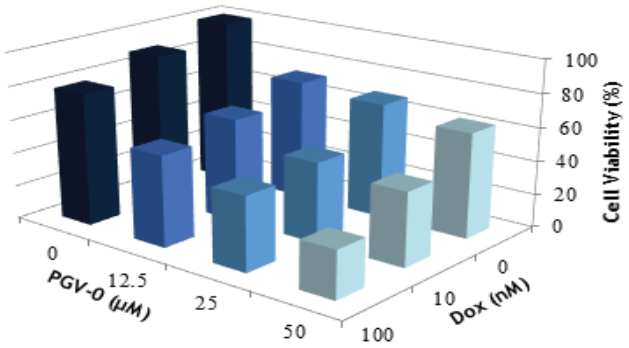

Figure 2. Cytotoxicity of Combination and single treatment on 4T1 cells. The 4T1 Cells (2.65x10^3 cell/well) were treated with combination of PGV-0 and doxorubicin (Dox) as described in the material and methods. After incubated for 24 h, the cells viability were measured using MTT assay.

cytotoxic assay, the data of single treatment PGV-0 (50 µM) of this experiment has higher death cells. We suspect this biased data may be occurred due to results of a long exposure of trypsin which resulted in leakage of cell metabolites (Bundscherer, *et al*., 2013). Regardless of these results, these data still give information that a combination of PGV-0 and doxorubicin may affect the apoptosis mechanism of doxorubicin on 4T1 cells.

Senescence Induction Effect of Combination PGV-0 and Doxorubicin

Besides apoptosis, another cellular mechanism that could be induced by anticancer compounds was senescence. Doxorubicin were known induce cellular senescence on cells. Therefore, these combinatioral treatment possibly affect senescence on 4T1. We were used SA-β-GAL assay to determine senescence cells. The senescence cells were identified by blue-greenish color to cellular senescence, while the untreated cells is given no change of color. Observation using an inverted microscope detected cellular senescence within single and combination treatment (Figure 4). Single treatment of PGV-0 at concentration 25 µM and 50 µM showed that PGV-0 induce senescence each 2.03% and 3.13 % on 4T1 cells. Meanwhile, combination treatment were exhibit senescence cells each 6.99 % and 7.70% higher than single treatment of doxorubicin 10 nM (3.33%). These data suggested that administration of PGV-0 could enhance senescence effect of doxorubicin on 4T1.

ROS Level of Single Treatment PGV-0 and Its Combination

The increase of apoptosis and senescence corresponding to an increase of cellular stress. The cancer cells has higher level of ROS compared with normal cells. However, an increase of ROS level could lead to oxydative stress which lead to both apoptosis and senescence. The combination of PGV-0 and doxorubicin may be increased ROS level on 4T1. Therefore to confirm these assumption, we also investigate ROS level on 4T1 cells. We were performed DCFDA staining assay to quantify ROS level on the 4T1 cells. The ROS level was calculated from fluorescence intensity given by the presence of DCF-fluorescence accumulated on cells. The single treatment of PGV-0 or doxorubicin increase ROS level, while combination of both compounds increases ROS level higher on 4T1 cells (Figure 5).

Table 1. Combination index (CI) analysis of doxorubicin with PGV-0. CI was used to determine interaction between each compound in combination treatment. Synergistic effect determined with CI value <0.9.

PGV-0	DOX (nM)	
(µM)	10	100
12.5	0.34	0.27
25	0.46	0.42
50	0.84	0.63

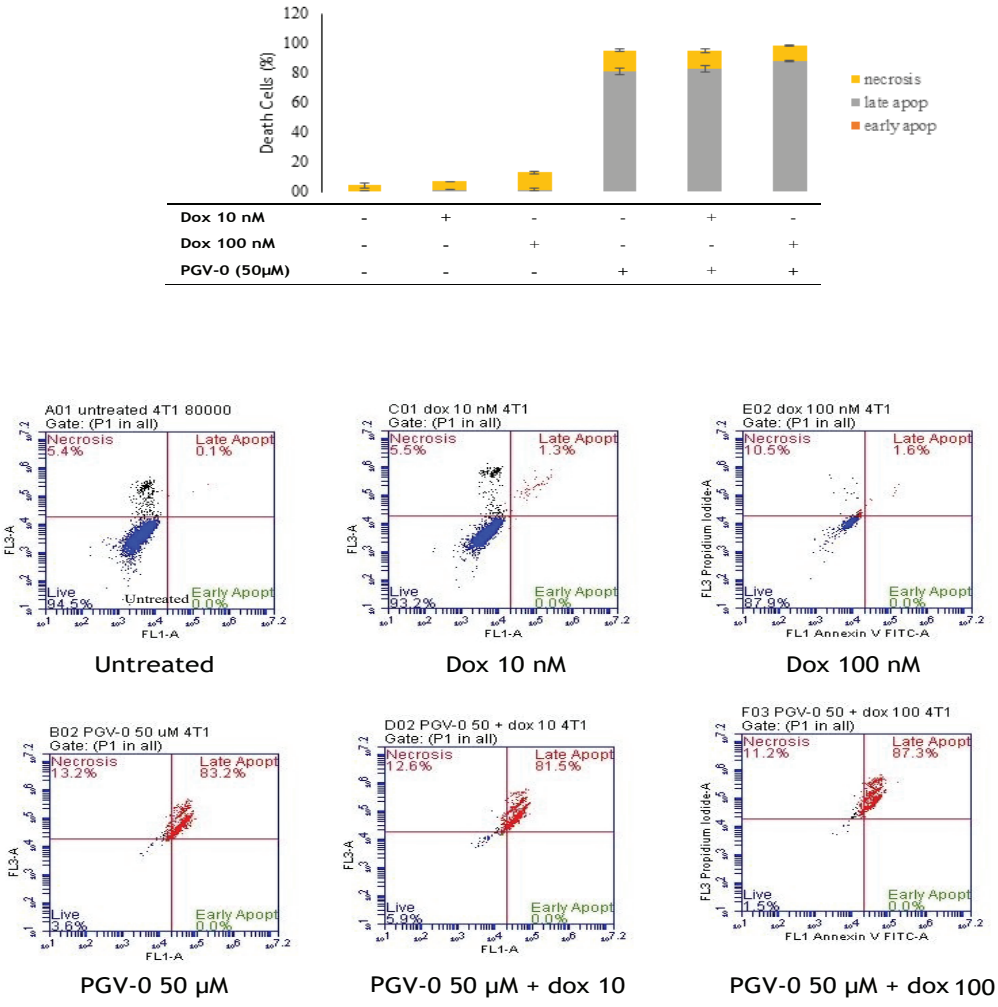

Figure 3. Apoptosis effect of PGV-0, doxorubicin and combination of both compound on 4T1 cells. Cells were treated with single treatment and combination of PGV-0 and doxorubicin. Treated cells were harvested and collected at microtube, then stained using annexin V/PI. The cells were analysed using flowcytometry (A). Quantification cell death in each group (B). Quadrant analysis of cells on flowcytometry.

Table 2. Quantification of apoptosis and necrosis using annexin V/PI staining.

Group	Early apoptosis (%)	Late apoptosis (%)	Necrosis (%)
Untreated	0.0	0.0	4.0
Dox 10 nM	0.0	1.3	5.5
Dox 100 nM	0.0	1.4	11.3
PGV-0 (50µM)	0.0	80.8	14.4
PGV-0 (50µM) + dox 10	0.0	82.8	11.8
PGV-0 (50µM) + dox 100	0.0	88.0	10.2

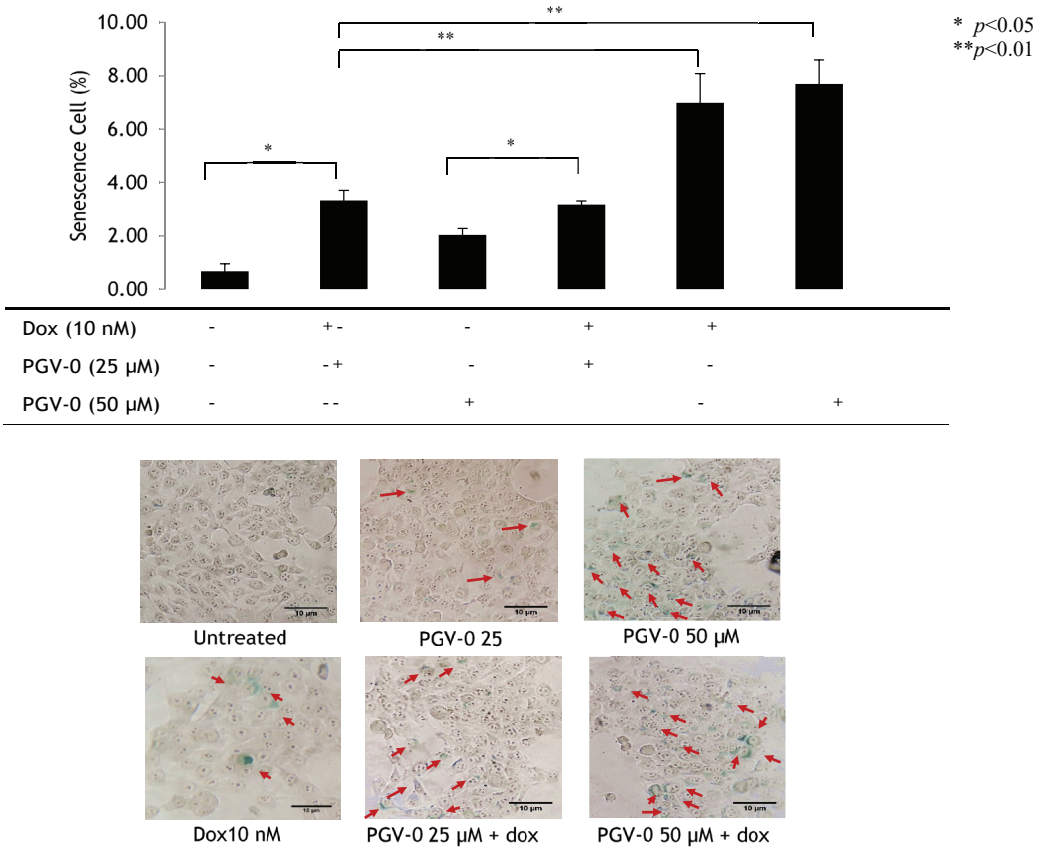

Figure 4. Senescence induction effect of PGV-0 singly and combination with doxorubicin. The 4T1 cell line ($1.5x10^6$ Cell/well) treated with single treatment of PGV-0 and combination with 10 nM doxorubicin. SA-β -GAL dye was given after 24 h treatment. Cellular senescence observed at 24, 48 and 72 h. (A) percentage of cellular senescence (n=3, *=p<0.05, **= p<0.01) (B). Morphology of cell after 72 h staining (scale bars =10 µm). Inverted microscope was used to observed cellular senescence (200X).

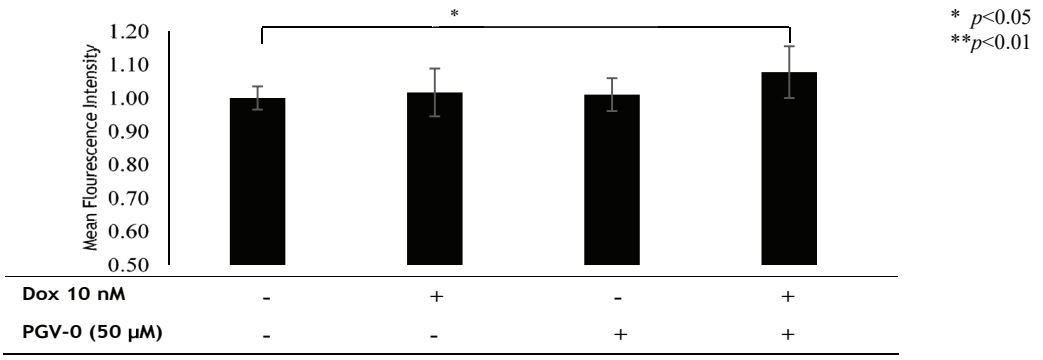

Figure 5. ROS level of 4T1 single treatment and combination PGV-0. Cells 4T1 culture ($5x10^4$ cell/well) were trypsinized and collected at microtube, then inactivated using supplemented buffer. DCFDA reagent (25 µM) were added to suspension cell. After that, suspension cells were treated with PGV-0 singly and its combination. After 4 h incubation, intensity ROS fluorescence analysed using flowcytometry instrument (*= p<0.05 **=p<0.01).

DISCUSSION

Doxorubicin has strong anticancer properties against breast cancer, thus making its usage more preferred for chemotherapy especially for TNBC (O'Reilly, *et al*., 2015). However, long term use of doxorubicin was restricted due to the adverse effect and risk of doxorubicin's resistance. Therefore, the exploration of co-chemotherapeutic agents urgently needed to improve the cytotoxicity of doxorubicin against cancer. This study aimed to explore the co-chemotherapeutic properties of PGV-0 on 4T1 cells as an *in vitro* model for TNBC.

PGV-0 compound is one of the potential curcumin analogs to be developed to improve the cytotoxicity of doxorubicin. Previously, PGV-0 reported form synergistic effect with doxorubicin which enhances the sensitivity of doxorubicin on MCF-7 resistance doxorubicin (MCF-7/Dox) (Meiyanto, *et al*., 2014). In this present study, we found that PGV-0 form synergistic effect which enhances the cytotoxicity of doxorubicin on 4T1 cells. Interestingly, investigation on cellular mechanism shows that PGV-0 also increases induction effects of doxorubicin on senescence, ROS and possibly apoptosis. Considering those data, it is likely that the inhibitory effect of PGV-0 may be correlated with the increase of oxidative stress through a higher level of ROS on 4T1 cells. Several studies reported that an increase of ROS over cellular threshold could trigger activation pathways of senescence and apoptosis, which led to the inducing of apoptosis and senescence on cells (Davalli, *et al*., 2016; Redza-Dutordoir and Averill-Bates, 2016). Taken those finding above, PGV-0 may enhance the cytotoxicity of doxorubicin through the increase of the ROS intracellular level. Nevertheless, the molecular mechanism of PGV-0 remains undiscovered yet. Further investigation is needed to prove this hypothesis as well as revealed the mechanism molecular of PGV-0.

CONCLUSION

Analog curcumin PGV-0 potential to be developed as a co-chemotherapeutic agent with doxorubicin against TNBC.

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