The Indonesian Biomedical Journal, Vol.15, No.5, October 2023, p.296-350 Print ISSN: 2085-3297, Online ISSN: 2355-9179

RESEARCH ARTICLE

# The Synergistic Cytotoxic Effect of Pentagamavunon-1 (PGV-1) and Curcumin Correlates with the Cell Cycle Arrest to Induce Mitotic Catastrophe in 4T1 and T47D Breast Cancer Cells

Desty Restia Rahmawati[1,2], Arief Nurrochmad[3], Riris Istighfari Jenie[2,4], Edy Meiyanto[2,4,*]

[1]Doctoral Program in Pharmaceutical Science, Faculty of Pharmacy, Universitas Gadjah Mada, Jl. Farmako, Sekip Utara, Yogyakarta 55281, Indonesia
[2]Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada, Jl. Farmako, Sekip Utara, Yogyakarta 55281, Indonesia
[3]Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Jl. Farmako, Sekip Utara, Yogyakarta 55281, Indonesia
[4]Laboratory of Macromolecular Engineering, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Jl. Farmako, Sekip Utara, Yogyakarta 55281, Indonesia

*Corresponding author. Email: edy_meiyanto@ugm.ac.id

Received date: Aug 10, 2023; Revised date: Sep 29, 2023; Accepted date: Oct 5, 2023

## Abstract

**BACKGROUND:** The anti-cancer properties of pentagamavunon-1 (PGV-1) and curcumin have been documented. This study aimed to evaluate the cytotoxic effect of this combination on breast cancer cell growth using 4T1 and T47D cells, representing triple-negative breast cancer (TNBC) and non-TNBC, respectively.

**METHODS:** Cytotoxic assay was evaluated using MTT reagent for single and combination treatment of PGV-1 and curcumin in 4T1 and T47D cells. Cell cycle analysis was examined through flowcytometry with propidium iodide dye. May Grünwald-Giemsa staining was also performed to analyze the mitotic catastrophe

**RESULTS:** PGV-1 and curcumin alone had significant cytotoxic effects against two breast cancer cell lines, with $IC_{50}$ values of 4 µM and 40 µM for 4T1 and 2 µM and 20 µM for T47D, respectively. Both compounds showed high selectivity for the 4T1 and T47D cells (selectivity index >3). In addition, when PGV-1 and curcumin were combined, a synergistic effect was observed in both cell types with a combination index of <0.7. This combination results in cell cycle arrest in the G2/M phase, increased cell accumulation in the sub-G1 phase, and a synergistic increase in mitotic catastrophe.

**CONCLUSION:** Combined intervention of PGV-1 and curcumin on TNBC and non-TNBC breast cancer cells substantially augments cell cycle arrest in the G2/M and sub-G1 phases, coupled with the occurrence of mitotic catastrophe. In summary, the results suggest that PGV-1 coupled with curcumin holds promise as an effective approach to addressing breast cancer and warrants further investigation.

**KEYWORDS:** 4T1 cells, T47D cells, breast cancer, curcumin, mitotic catastrophe, PGV-1

***Indones Biomed J. 2023; 15(5): 318-27***

## Introduction

Pentagamavunon-1 (PGV-1), a curcumin derivative, shows potential as a promising anti-cancer agent.(1) Curcumin, a polyphenol from *Curcuma* species (2) has proven anti-cancer properties on various cancer cells, including breast, colon, and blood cancer.(3,4) PGV-1 has better physicochemical stability and significant anti-cancer activity, especially in breast cancer cells.(5) The presence of PGV-1 as a curcumin derivative introduces prospects for increasing the efficacy of anti-cancer treatments, especially in breast cancer. This potential augmentation promises to overcome challenges such as the adverse

effects and drug resistance associated with conventional chemotherapy regimens.(5–7)

The pivotal advantage of curcumin, as a potential co-agent alongside PGV-1, lies in its capacity to modulate the cell cycle.(8) The strategy of cell cycle modulation gains traction in cancer treatment due to the frequently disrupted cell cycle regulation observed in cancer cells, leading to uncontrolled proliferation.(9–11) Previous studies have demonstrated a synergistic effect of PGV-1 in combination with natural compounds such as piperine, galangin, and diosmin against breast cancer cells.(12–14) Nevertheless, the cytotoxic effect of this combination remains relatively modest. Hence, the amalgamation of PGV-1 with curcumin, which boasts robust cytotoxic properties, holds the promise of enhancing the cytotoxic impact of the combination, resulting in a more robust antineoplastic response.(15) Although PGV-1 and curcumin share similarities in their mechanism of action as anti-cancer agents, they differ in the mechanisms and targets they affect. PGV-1 exerts its growth inhibitory effect by targeting prometaphase, whereas curcumin concentrates its activity on the G2/M phase.(1,8) Consequently, the simultaneous utilization of these compounds is expected to exert a broader and more profound influence on the cell cycle, thereby amplifying the impact on apoptosis of cancer cells.

This study has an important impact on managing difficult-to-treat breast cancer, especially triple-negative breast cancer (TNBC) and non-TNBC. Breast cancer is a complex global challenge.(16,17) Focus on developing treatment solutions, especially for TNBC, is critical due to its aggressive nature and limited treatment options.(18) Likewise, non-TNBC also has a high incidence rate.(19) Therefore, further research regarding the potential of PGV-1 and curcumin in facing the challenges of breast cancer, both TNBC and non-TNBC, is the main focus. The combination of the two is expected to provide new understanding in overcoming drug resistance and responding more strongly to cancer cell growth. By understanding its in-depth mechanism of action, this can help the development of more effective and selective drugs, bringing new hope to the medical world as a whole. Therefore, it is important to investigate in depth how the combination of PGV-1 and curcumin affects breast cancer cells, both TNBC and non-TNBC. The compelling rationale underlying the combination of PGV-1 and curcumin is to attain a synergistic effect, thereby heightening the anti-cancer potency of PGV-1. Their combined application can synergistically impact perturbing the cell cycle regulation of cancer cells, potentially triggering a more potent apoptotic response. This research has the potential to become a solid scientific basis for developing more effective and innovative therapies in addressing the challenges of breast cancer, which has complex and diverse characteristics. The main objective of this study was to evaluate the cytotoxic effect produced by the combination of PGV-1 and curcumin, particularly in inhibiting breast cancer cell proliferation. Treatment strategies vary greatly when comparing non-TNBC and TNBC breast cancer. Non-TNBC, which includes cases with different receptors such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), may be treated in a targeted manner, tailoring therapy to suit the characteristics of the target receptors. In contrast, TNBC does not have a clear target receptor, making treatment more challenging because there is no specific focal point for intervention. Two cell models were used in this study: the 4T1 model represents TNBC cells and the T47D model represents non-TNBC cells. This study aimed to comprehensively understand the effect of the combination tested on these two breast cancer cell subtypes.

## Methods

### Cell Culture

4T1 (ATCC no. CRL-2539™) and T47D (ATCC no. HTB-133™) breast cancer cells, as well as normal cells, including Vero (ATCC no. CCL-81™) and NIH-3T3 (ATCC no. CRL-1658™) fibroblast cells, were obtained from Prof. Masashi Kawaichi at the Nara Institute of Science and Technology, Japan. 4T1, Vero, and NIH-3T3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA), while T47D cells were maintained in Roswell Park Memorial Institute (RPMI) medium (Gibco). These media were high glucose culture media supplemented with 10% (v/v) fetal bovine serum (FBS), HEPES (Sigma, St. Louis, MO, USA), and sodium bicarbonate (Sigma). Cells were stored in an incubator under humid conditions at 37°C with 5% $CO_2$.

### Cytotoxic Assay

Cytotoxic activity of PGV-1 and curcumin, both alone and in combination, was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.(20,21) 4T1 ($3.5\times10^3$ cells/well), T47D ($5\times10^3$ cells/well), Vero ($7\times10^3$ cells/well), and NIH-3T3 cells ($7\times10^3$ cells/well) were placed in 96-well plates and incubated for 24 hours until reached confluence of 80%. Then, the cells were treated individually with 0.1-10 µM

PGV-1 (synthesized by Cancer Chemoprevention Research Center (CCRC) Universitas Gadjah Mada) and 1-100 µM curcumin (synthesized by CCRC Universitas Gadjah Mada). Treatment was carried out in culture media for 24 hours. Next, the cells were incubated for 4 hours in MTT solution (Sigma) and diluted in the appropriate culture medium until formazan crystals formed. The reaction was stopped by the addition of 10% sodium dodecyl sulfate (SDS) (Merck, Darmstadt, Germany) in 0.01 N HCl (Merck), and the samples were then stored protected from light at room temperature overnight. Finally, the absorption in each well was measured using a microplate reader (BioRad, Hercules, CA, USA) at a wavelength of 595 nm, with data collection carried out in three replications (n=3) and the $IC_{50}$ value was calculated.

The selectivity index (SI) was calculated from the $IC_{50}$ of PGV-1 and curcumin in normal cells and cancer cells. The SI value >3 indicates the compound has a high selectivity. For the combined MTT test, the $IC_{50}$ value of the single treatment determined the concentration to be applied. Each compound has three concentration series, namely ⅛, ¼, dan ½ $IC_{50}$. Chou-Talalay method (22) was used to calculate the combination index (CI) value, as previously reported. The CI value classification is CI <1, =1, and >1, indicating synergism, additive effect, and antagonism, respectively.

**Cell Cycle**

The 4T1 ($2.5\times10^5$ cells/well) and T47D cells ($3\times10^5$ cells/well) were treated with PGV-1 and curcumin at ¼ and ½ $IC_{50}$, either single or in combination for 24 hours. The cells were then harvested through trypsinization, washed with phosphate-buffered saline (PBS) 1×, and centrifuged at 2,500 rpm for 5 minutes. The cells were fixed using 70% ethanol and stained with a PI/RNase Staining Buffer solution (BD Biosciences, Franklin Lakes, NJ, USA), as per the manufacturer's instructions, and 0.1% Triton X-100 was added. BD Accuri™ C6 Flow Cytometer (BD Bioscience) was used to analyze the fluorescence of the DNA-propidium iodide (PI) complex in the cells.

**Mitotic Catastrophe**

May-Grünwald Giemsa staining was used to investigate the effect of a combination of PGV-1 and curcumin in influencing cell cycle arrest, especially in the mitotic phase, in 4T1 and T47D cells. 4T1 cells ($5\times10^4$ cells/well) and T47D cells ($7\times10^4$ cells/well) were cultured on 6-well plates, incubated for 24 hours, and allowed to grow until they reached 80% confluency. A series of experiments were carried out with various concentrations of PGV-1 and single curcumin, as well as a combination of both, and then incubated for 24 hours. The next step was to stain by removing the media in the wells and washing with PBS 1× twice. Then, the cells were stained with May-Grünwald Giemsa stain (Merck), incubated for 5 minutes, and washed with a phosphate buffer pH 7.2 solution once for 1.5 minutes. Subsequently, 500 µL/well of Giemsa-phosphate buffer (1:50) solution was added and allowed to stand for 15-20 minutes at room temperature. After that, the Giemsa solution was discarded, the cells were washed quickly with distilled water, and dried. Observations were performed using an inverted microscope (Olympus, Tokyo, Japan), and images were taken using a camera with three fields of view per treatment. The analysis was carried out by observing the number of cells that form micronuclei or polynuclei. The observations of cell morphology were carried out by two observers who did not know the treatment given.

**Statistical Analysis**

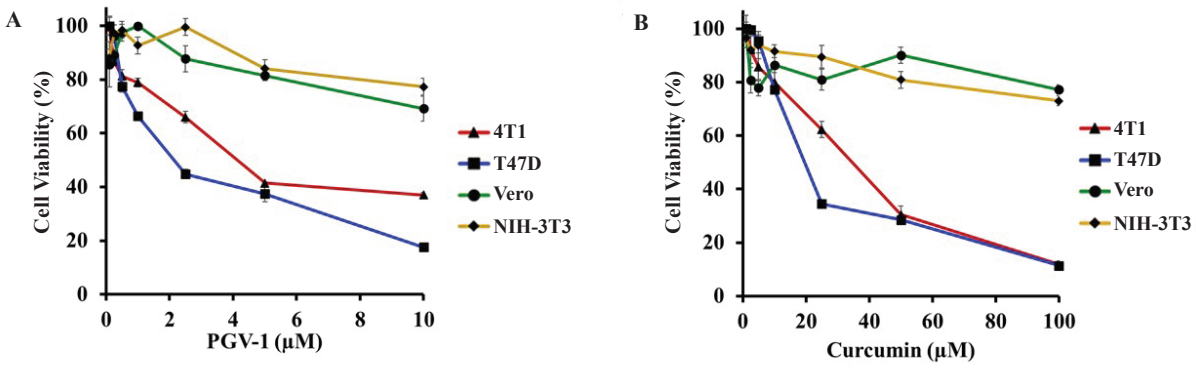
The results were presented as mean ± SD and statistically analyzed using SPSS 25 (IBM, Armonk, NY, USA). For the significance of differences between the two experimental conditions, a Student's t-test was employed. The significance of the differences was indicated by *p*-values, with asterisks representing the degree of significance (*$p$<0.05, **$p$<0.01, ***$p$<0.001, ****$p$<0.0001).

## Results

**Cytotoxic Effects and Selectivity of PGV-1 and Curcumin on 4T1 and T47D Cells**

To assess the cytotoxic effect of the combination of PGV-1 and curcumin, initially individual cytotoxicity assessments were conducted utilizing the MTT assay at concentration ranges of 0.1–10 µM for PGV-1 and 1–100 µM for curcumin on 4T1 and T47D cells (Figure 1A, Figure 1B). The results showed that PGV-1 cytotoxicity was more effective on T47D cells than on 4T1 cells, with $IC_{50}$ values of 2 µM and 4 µM, respectively. In 4T1 cells, PGV-1 showed a more potent cytotoxic effect than curcumin, with an $IC_{50}$ value of 4 µM for PGV-1 and 40 µM for curcumin. Moreover, the cytotoxicity of PGV-1 in T47D was also observed. This luminal breast cancer expressed $ER^+$ and $PR^+$ and showed a more potent growth suppression effect when treated with PGV-1 than curcumin, with IC50 values of 2 µM and 20 µM for PGV-1 and curcumin, respectively (Figure 1C).

The cytotoxicity of the compounds on normal cells (Vero and NIH-3T3) was evaluated using the MTT test at




C

| Compound | $IC_{50}$ (µM) | | | |
|---|---|---|---|---|
| | 4T1 | T47D | Vero | NIH-3T3 |
| PGV-1 | 4 | 2 | >10 | >10 |
| Curcumin | 40 | 20 | >100 | >100 |

D

| Compound | Selectivity Index (SI) | | | |
|---|---|---|---|---|
| | Vero | | NIH-3T3 | |
| | 4T1 | T47D | 4T1 | T47D |
| PGV-1 | 3.95 | 8.54 | 5.46 | 11.79 |
| Curcumin | 11.89 | 24.42 | 4.80 | 9.86 |

**Figure 1. Cytotoxic effects of PGV-1 and curcumin on 4T1, T47D, Vero, and NIH-3T3.** Cell viability was determined by MTT assay, cells were incubated with the compounds for 24 hours. A: Cell viability profiles after treatment with PGV-1: B: Cell viability profiles after treatment with curcumin; C: The IC50 values of PGV-1 and curcumin from each cell. D: The selectivity index of PGV-1 and curcumin on 4T1 and T47D cells against Vero and NIH-3T3 cells.

the same concentrations. The results showed that PGV-1 treatment on Vero and NIH-3T3 cells at concentrations up to 10 µM still provided cell viability above 80% (Figure 1A). Similarly, treatment of both normal cells with curcumin at concentrations up to 100 µM still demonstrated cell viability above 80% (Figure 1B). These findings suggested that curcumin and PGV-1 were not toxic to normal cells. Additionally, by contrasting the $IC_{50}$ ratio of normal cells to cancer cells, it was possible to ascertain the selectivity of the two compounds. According to the findings, PGV-1 was selective for 4T1 and T47D cancer cells, with SI values for Vero cells of 3.95 and 8.54 and NIH-3T3 cells of 5.46 and 11.79, respectively. Similarly, treatment with curcumin showed SI values for 4T1 and T47D cells of 11.89 and 24.42 (Vero) and 4.80 and 9.86 (NIH-3T3), respectively (Figure 1D). The SI values of PGV-1 and curcumin treatment were >3, so it can be concluded that these compound had high selectivity in both types of cancer cells tested.

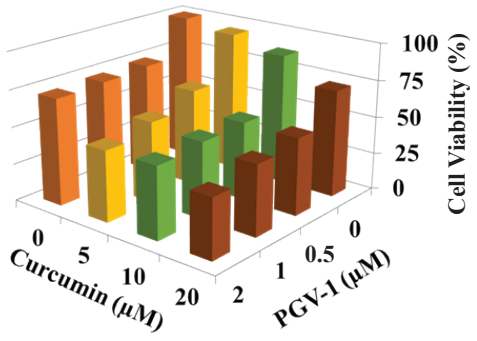
### PGV-1 and Curcumin Synergistically Suppressed the Growth of Breast Cancer Cells

The cytotoxic effect of the combination of PGV-1 and curcumin was determined by treating the cells with ⅛, ¼, and ½ of $IC_{50}$ concentrations for each component. The CI value was used to assess cytotoxicity. Interestingly, the combination treatment reduced the cell viability of 4T1 and T47D cells better than the single treatment, as shown by all combination treatments (Figure 2A, Figure 2B). The combination treatment reduced cell viability with CI values less than 0.7 (Figure 2C, Figure 2D). This finding indicated that curcumin provided a synergistic effect in enhancing the cytotoxicity of PGV-1 toward breast cancer cells.

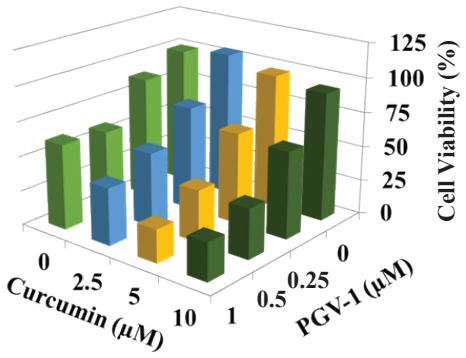
### PGV-1 and Curcumin Induced G2/M Arrest on 4T1 and T47D Cells

To examine the impact of the incorporation of PGV-1 and curcumin on cell cycle progression, flow cytometry analysis was performed on 4T1 and T47D cells after treatment (Figure 3A, Figure 3C). The results showed that when applied as a single treatment on 4T1, PGV-1 significantly increased cell accumulation in the G2/M phase ($p$<0.0001), and curcumin at a concentration of ½ of $IC_{50}$ (20 µM) significantly increased cell accumulation in the G2/M phase ($p$<0.0001) but not at the concentration of ¼ of $IC_{50}$ (10 µM). However, when PGV-1 combined with curcumin, the cell cycle profile changed significantly to increase accumulation in the sub-G1 phase compared to the single treatments ($p$<0.0001), indicating the increase of cell death. However, no significant effect was observed with the combination of ¼ of $IC_{50}$ (1 µM) of PGV-1. Meanwhile, the combination of ½ of the $IC_{50}$ (1 µM) of PGV-1 and ½ of the $IC_{50}$ (10 µM) of curcumin was effective to induce sub-G1 phase significantly ($p$<0.0001) on T47D cells. The results suggest that the synergistic effect of the combination of

A




B




C

| Combination Index (CI) | | | |
|---|---|---|---|
| Curcumin (μM) | PGV-1 (μM) | | |
| | 0.5 | 1 | 2 |
| 5 | 0.32 | 0.31 | 0.41 |
| 10 | 0.35 | 0.42 | 0.54 |
| 20 | 0.60 | 0.60 | 0.64 |

D

| Combination Index (CI) | | | |
|---|---|---|---|
| Curcumin (μM) | PGV-1 (μM) | | |
| | 0.25 | 0.5 | 1 |
| 2.5 | 0.27 | 0.18 | 0.24 |
| 5 | 0.27 | 0.18 | 0.21 |
| 10 | 0.42 | 0.26 | 0.30 |

**Figure 2. Combination cytotoxic effect of PGV-1 and curcumin on 4T1 and T47D cells.** The combination index (CI) of 4T1 and T47D cells was calculated by using the percent viability of cells treated with PGV-1 and curcumin both individually and in combination. A: The profile of cell viability after treatment with a combination of PGV-1 and curcumin in 4T1 cells; B: The profile of cell viability after treatment with a combination of PGV-1 and curcumin in T47D cells. C: CI of 4T1 cells; D: CI of T47D cells.

PGV-1 and curcumin may be associated with an increase in the sub-G1 population and possibly cell cycle arrest in the G2/M phase, compared with the use of PGV-1 alone in both cells. The results showed that PGV-1 alone or combined with curcumin can cause cell cycle arrest, thus allowing increased apoptosis (Figure 3B, Figure 3D).

### The Mitotic Catastrophe Induction of PGV-1 and Curcumin on 4T1 and T47D Cells

The mitotic catastrophe was measured to determine the specific arrest point of PGV-1 in the cell cycle. Therefore, the combination of PGV-1 and curcumin on the specific effect of mitotic arrest in 4T1 and T47D cells was tested using May Grünwald-Giemsa staining. As in previous cell cycle tests, both cells were treated with two concentrations below $IC_{50}$ of each compound for 24 hours. May–Grünwald Giemsa-stained cells can be distinguished from the G1/S/G2 phase into the mitotic phase by evident nucleus loss or the occurrence of mitotic catastrophe.(1) Figure 4A and Figure 4B showed that PGV-1 treatment alone dramatically enhanced the number of cells with mitotic catastrophe and, more precisely, showed that cells were arrested at the prometaphase stage.

The test results showed that single PGV-1 had a much higher catastrophic effect than single curcumin on 4T1 cells ($p$<0.05) and T47D cells ($p$<0.01). The results depicted that single PGV-1 produced cell enlargement with chromosome condensation as an indication of mitotic arrest and mitotic catastrophe phenomenon. Conversely, curcumin induced partial condensation of normal-sized chromosomes representing mitotic arrest without cellular catastrophe. Interestingly, curcumin significantly increased condensation in 4T1 cells ($p$<0.001) and T47D cells ($p$<0.01) compared with the untreated cells. The results showed that when PGV-1 was combined with curcumin, it was able to increase the incidence of catastrophic mitosis significantly compared with PGV-1 single treatment at all concentrations tested in 4T1 cells ($p$<0.05). In T47D cells, PGV-1 combined with curcumin was able to significantly increase the incidence of mitotic catastrophe ($p$<0.01) compared to single treatment of PGV-1, which may accelerate cellular death events (4). Thus, curcumin contributes to the effectiveness of permanently enhancing the PGV-1 mitotic arrest in 4T1 and T47D cells. Therefore, this indicated that the combination treatment was more effective in increasing cell cycle cessation at the mitosis phase, causing the cells to experience a cellular catastrophe, leading to cancer cell death.

## Discussion

Previous studies revealed that PGV-1 had the potential to inhibit the growth of breast cancer cells, both TNBC and non-TNBC types. Previously, curcumin has been found to cause apoptosis in luminal type A breast cancer cells.(5) Other studies also state that curcumin can affect reactive

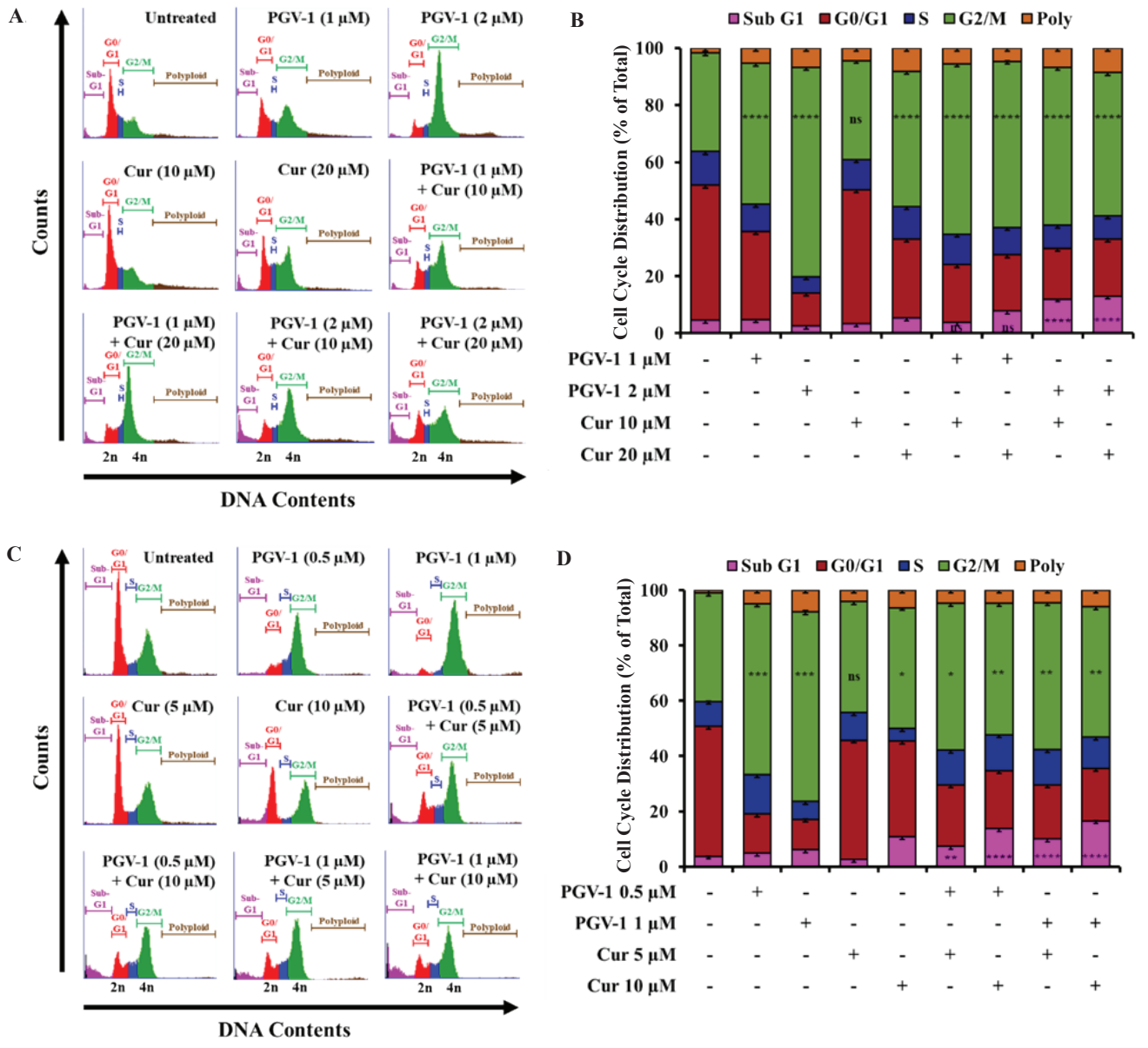



**Figure 3. Effects of PGV-1 and curcumin on the cell cycle profiles of 4T1 and T47D cells.** A: Flow cytograms of each treatment of 4T1 cells; B: Quantification of 4T1 cell population of each phase; C: Flow cytograms of each treatment of T47D cells; D: Quantification of T47D cell population of each phase. The significant analysis shown the comparation on G2/M phase of the single and combination treatment with the control group and the comparation on sub-G1 phase of combination treatment with single treatment. *$p$<0.05; **$p$<0.01; ***$p$<0.001; ****$p$<0.0001; ns: not significant.

oxygen species (ROS) production. When cells are exposed to curcumin, ROS production increases, which is associated with the induction of apoptosis.(1,8) This study focused on cell accumulation in different cell cycle phases because it is well known that cell cycle progression is a target for PGV-1 toxicity.(1)

In this experiment, curcumin was described as a combination agent for PGV-1. Previous studies have shown that both can inhibit the growth of TNBC and non-TNBC cells.(12,23,24) Curcumin had about 2 times stronger anti-proliferative activity on T47D cells ($IC_{50}$=20 µM) than on 4T1 cells ($IC_{50}$=40 µM) (Figure 1C). PGV-1 showed a more substantial inhibitory effect on the proliferation of T47D cells than 4T1 cells. Interestingly, combination of PGV-1 and curcumin produced a better synergistic effect in reducing the viability of 4T1 and T47D cells compared to each compound separately (CI<0.7). In TNBC and non-TNBC cell cycle profiles, PGV-1 supplemented with curcumin could increase cell cycle arrest in the G2/M phase, which aligned with the increase in the sub-G1 phase associated with programmed

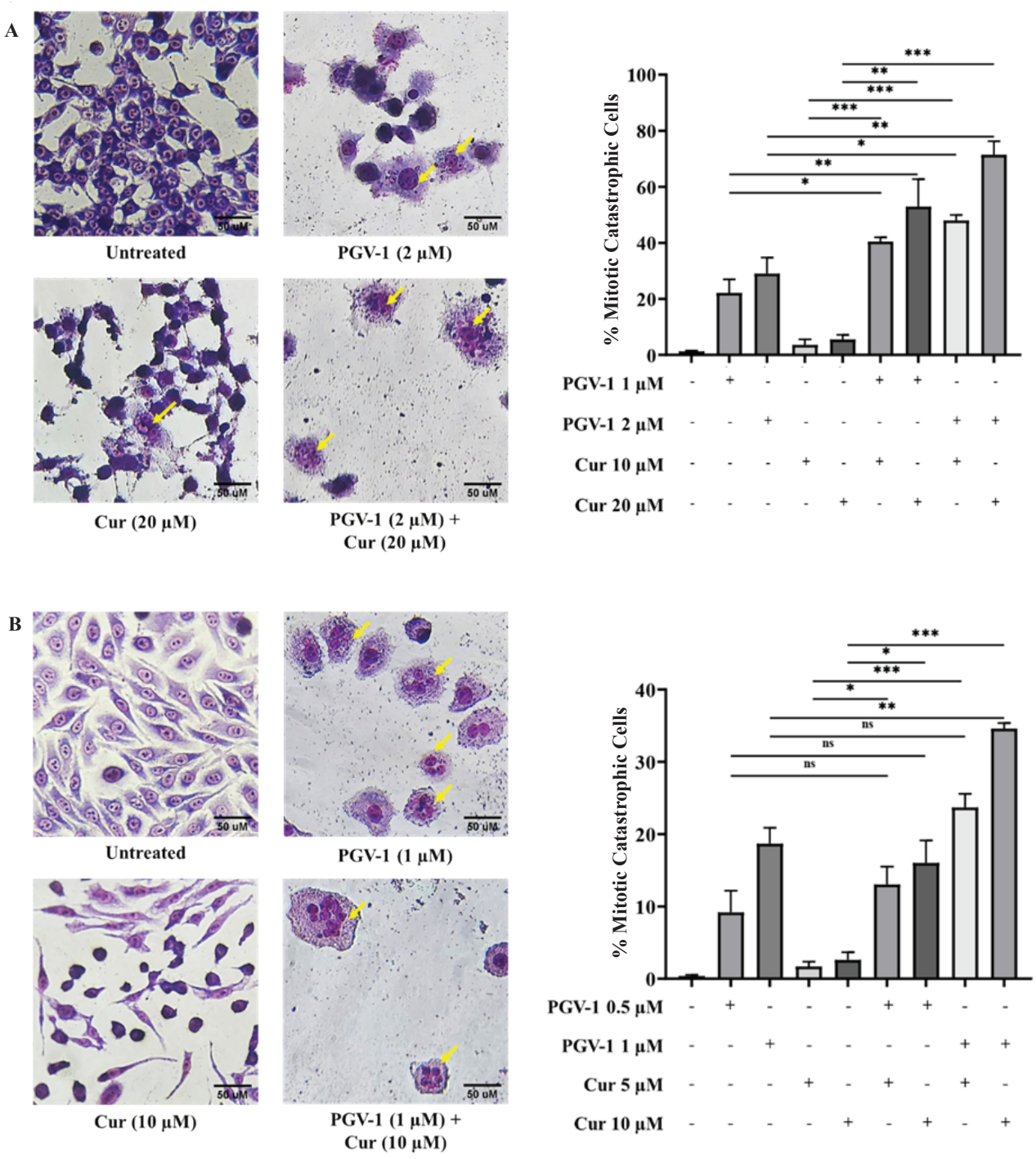



**Figure 4. Mitotic catastrophe induction effect of PGV-1 and curcumin on 4T1 and T47D cells.** The proportion of mitotic catastrophe cells was determined by microscopic analysis. A, left panel: The morphology of 4T1 cells; A, right panel: The proportion of mitotic catastrophic 4T1 cells; B, left panel: The morphology of T47D cells; B, right panel: The proportion of mitotic catastrophic T47D cells. Yellow arrows indicate polynuclear cells/mitotic catastrophe. *$p$<0.05; **$p$<0.01; ***$p$<0.001; ****$p$<0.0001; ns: not significant.

cell death. PGV-1 individually inhibits cell cycle growth in the G2/M phase of the cell cycle in several cancer cell lines, such as breast, colon, and blood.(1,4–6) This fact is corroborated by evidence that PGV-1 removes cyclins A, B, and D.(4) The present study also showed that both PGV-1 and curcumin were safe for normal cells. In addition, by comparing the IC50 ratio in normal cells and cancer cells, the selectivity of the two compounds can be confirmed. With the SI value of PGV-1 and curcumin treatment more than 3, it can be concluded that the two compounds have high selectivity for the two types of cancer cells tested. A previous study showed that PGV-1 was more selective

against normal HEK293T cells than MCF-7 breast cancer cells, with a selectivity index (SI) score of 5.57.(23) PGV-1 also exhibits good stability properties. Treatment with PGV-1 remained effective on K562 leukemic cells after 6 days without any measurable compounds, with cell viability of less than 5%.(1) This suggests that PGV-1 irreversibly prevents cancer cell proliferation. However, co-treatment with curcumin on breast cancer cells and normal cells requires further investigation regarding their effectiveness over time and safety assurance.

Inhibition of mitotic catastrophe is the main goal in the fight against cancer cells, especially breast cancer. PGV-1 can inhibit the cell cycle in the G2/M phase, especially in prometaphase.(1) This finding is consistent with the results of the May Grünwald-Giemsa stain, which demonstrated that PGV-1 and its combination with curcumin were effective in inhibiting the cell cycle in the mitotic phase through mitotic catastrophe events in 4T1 and T47D cells. Mitotic catastrophe is defined by characteristic changes in the nucleus and the appearance of multinucleated giant cells.(12) Multinucleated giant cells undergo a process of gradual death through mechanisms involving apoptosis and necrosis.(25,26) These signs were seen in 4T1 and T47D cells after treatment with 1 µM and 0.5 µM PGV-1, respectively. However, the highest incidence of mitotic catastrophe occurred in 4T1 cells when given a combination of 2 µM PGV-1 with 20 µM curcumin and in T47D cells when given a combination of 1 µM PGV-1 with 5 µM and 10 µM curcumin. Most cells that undergo mitotic catastrophe during the prometaphase stage eventually die. It is established that PGV-1 distinctly triggers a state of prometaphase arrest during the M phase, whereas curcumin exerts inhibitory influences across multiple junctures within the cell cycle.(1,8) The cell cycle inhibitory effect of PGV-1 on prometaphase causes cellular processes that result in permanent growth atrophy, known as senescence. Induction of senescence prevents cells from returning to the cell cycle and leads to cell death.(27)

Treatment with PGV-1 and curcumin was more effective on non-TNBC cells (T47D) compared to TNBC cells (4T1) as shown in the cytotoxic (Figure 1) and cell cycle results (Figure 3). This may be due to several factors. TNBC cells tend to be more aggressive and challenging to treat due to the lack of specific hormone receptors (ER, PR, HER2).(28) In contrast, T47D cells are non-TNBC cells, thus providing an additional target for more effective treatment. Curcumin has been studied for its anti-inflammatory, antioxidant, and anti-cancer potential.(29,30) T47D cells which have been known to have a richer receptor profile could respond better to curcumin's anti-cancer effects. (31,32) There may also be different molecular interactions between PGV-1 and 4T1 cells as well as T47D cells and distinct signaling pathways specific to T47D cells that respond more strongly to the combination of PGV-1 and curcumin than 4T1 cells. TNBC (4T1 cells) and non-TNBC (T47D cells) could have different sensitivities to certain compounds. Perhaps T47D cells are more susceptible to a combination of PGV-1 and curcumin than 4T1 cells, and it is evident from the results of the cytotoxic profile that the effect of PGV-1 and curcumin in T47D cells were stronger (lower $IC_{50}$) than in 4T1 cells. T47D cells may have cellular activities that are more easily disrupted by the combination of PGV-1 and curcumin, such as cell cycle inhibition and apoptosis, leading to a more significant impact on cancer growth.

Mitotic catastrophe, which can be seen through the May Grünwald-Giemsa stain, is characterized by abundant micronuclei and mitotic arrest.(25) Micronuclei are formed when the chromosome fragments are not evenly distributed in the nucleus, resulting in non-uniform nuclei due to karyokinetic imbalance. Multinucleated cells also arise because chromosomes do not condense during division. (26) Checkpoint mechanisms fail to halt mitotic progress and resist adverse effects until DNA repair occurs, causing a mitotic catastrophe. Inhibition of G2/M checker genes, pharmacological or genetic, increases the chances of mitotic catastrophe.(25,26) This is in line with the mechanism of action of PGV-1 and curcumin, which inhibits the G2/M phase of the cell cycle.(1,8) Curcumin, alone or in combination with PGV-1, only affected G2/M arrest. The results might be more effective on non-TNBC (T47D) cells with PGV-1 and curcumin. PGV-1 has anti-cancer properties, although modest. PGV-1 has been reported to induce mitotic arrest in K562 cells.(1) In this case, PGV-1 alone induces prometaphase arrest, whereas curcumin inhibit cell growth in G2/M phase. This combination is interesting to be explored further especially for the molecular target of PGV-1 in the mitotic regulator such as polo like kinase 1 (PLK1), and curcumin should be targeted in G1 regulation, such as cyclin-B expression and nuclear factor-kappa B (NF-κB) activation. Curcumin increased the anti-cancer potential of PGV-1 towards TNBC and non-TNBC breast cancer. Combination of PGV-1 and curcumin significantly increased cell death, especially induction of G2/M and sub-G1 cell cycle arrest, and increased mitotic catastrophe. Co-administration of PGV-1 and curcumin could be a practical therapeutic approach for TNBC and non-TNBC.

Overall, the study demonstrated that PGV-1 possesses anti-cancer properties, and its combination with curcumin enhanced its effectiveness in treating both TNBC and non-TNBC breast cancer. Synergistic effect of the combination resulted in increased cell death by inducing cell cycle arrest and mitotic catastrophe. These findings suggest that the concurrent administration of PGV-1 and curcumin could be a promising therapeutic approach for breast cancer treatment.

## Conclusion

This study revealed that PGV-1 has promising anti-breast cancer potential, although it is still relatively low. When PGV-1 and curcumin are combined, they work synergistically to suppress cell growth. This co-treatment process also induces cell cycle arrest in the G2/M and sub-G1 phases, triggering an increase in the mitotic catastrophe phase. The results of this study indicate that the use of PGV-1 and curcumin together can be an effective therapy in treating both TNBC and non-TNBC.

## Acknowledgments

This research was funded by the Education of Master Degree Leading to Doctoral Program for Excellent Graduates (PMDSU), Ministry of Education, Culture, Research, and Technology, Republic of Indonesia (Grant Number: 1985/UN1/DITLIT/Dit-Lit/PT.01.03/2022). The authors also thank the National Research and Innovation Agency (BRIN), Research and Development of Medicinal Plants and Traditional Medicine Center (B2P2TOOT), Tawangmangu, Karanganyar, Indonesia, which has facilitated the flow-cytometry analysis.

## Authors Contribution

AN and EM were responsible for generating the research idea and conceptualizing the study. DRR conducted the experiments and performed data analysis. The initial draft of the research article was prepared by DRR and RIJ. EM, AN, and RIJ provided oversight and significant contributions to refining the research conception. Collaboratively, DRR, AN, EM, and RIJ completed the manuscript. The final manuscript was read and approved by all authors.

## References

1. Lestari B, Nakamae I, Yoneda-Kato N, Morimoto T, Kanaya S, Yokoyama T, *et al*. Pentagamavunon-1 (PGV-1) inhibits ROS metabolic enzymes and suppresses tumor cell growth by inducing M phase (prometaphase) arrest and cell senescence. Sci Rep. 2019; 9(1): 14867. doi: 10.1038/s41598-019-51244-3.
2. Pujimulyani D, Yulianto WA, Setyawati A, Rizal R, Qodariah RL, Khoiriyah Z, *et al*. Curcuma mangga Val. extract as antidiabetic agent in 3T3-L1 adipocyte cells. Mol Cell Biomed Sci. 2020; 4(1): 45-51.
3. Shankar S, Srivastava RK. Bax and Bak genes are essential for maximum apoptotic response by curcumin, a polyphenolic compound and cancer chemopreventive agent derived from turmeric, Curcuma longa. Carcinogenesis. 2007; 28(6): 1277-86.
4. Meiyanto E, Septisetyani EP, Larasati YA, Kawaichi M. Curcumin analog pentagamavunon-1 (PGV-1) sensitizes Widr cells to 5-fluorouracil through inhibition of NF-κB activation. Asian Pac J Cancer Prev. 2018; 19(1): 49-56.
5. Meiyanto E, Putri DD, Susidarti RA, Murwanti R, Sardjiman, Fitriasari A, *et al*. Curcumin and its analogues (PGV-0 and PGV-1) enhance sensitivity of resistant MCF-7 cells to doxorubicin through inhibition of HER2 and NF-kB activation. Asian Pac J Cancer Prev. 2014; 15(1): 179-84.
6. Novitasari D, Jenie RI, Utomo RY, Kato JY, Meiyanto E. CCA-1.1, a novel curcumin analog, exerts cytotoxic anti-migratory activity toward TNBC and HER2-enriched breast cancer cells. Asian Pac J Cancer Prev. 2021; 22(6): 1827-36.
7. Wulandari F, Kirihata M, Kato JY, Meiyanto E. Curcumin analogs, PGV-1 and CCA-1.1 exhibit anti-migratory effects and suppress MMP9 expression on WiDr cells. Indones Biomed J. 2021; 13(3): 271-80.
8. Larasati YA, Yoneda-Kato N, Nakamae I, Yokoyama T, Meiyanto E, Kato JY. Curcumin targets multiple enzymes involved in the ROS metabolic pathway to suppress tumor cell growth. Sci Rep. 2018; 8(1): 2039. doi: 10.1038/s41598-018-20179-6.
9. Scott SJ, Li X, Jammula S, Devonshire G, Lindon C, Fitzgerald RC, *et al*. Evidence that polyploidy in esophageal adenocarcinoma originates from mitotic slippage caused by defective chromosome attachments. Cell Death Differ. 2021; 28(7): 2179-93.
10. Feillet C, van der Horst GT, Levi F, Rand DA, Delaunay F. Coupling between the circadian clock and cell cycle oscillators: Implication for healthy cells and malignant growth. Front Neurol. 2015; 6: 96. doi: 10.3389/fneur.2015.00096.
11. Blagosklonny MV, Pardee AB. The restriction point of the cell cycle. Cell Cycle. 2002; 1(2): 103-10.
12. Endah E, Wulandari F, Putri Y, Jenie RI, Meiyanto E. Piperine increases pentagamavunon-1 anti-cancer activity on 4T1 breast cancer through mitotic catastrophe mechanism and senescence with sharing targeting on mitotic regulatory proteins. Iran J Pharm Res. 2022; 21(1): e123820. doi: 10.5812/ijpr.123820.
13. Musyayyadah H, Wulandari F, Nangimi AF, Anggraeni AD, Ikawati M, Meiyanto E. The growth suppression activity of diosmin and PGV-1 co-treatment on 4T1 breast cancer targets mitotic regulatory proteins. Asian Pac J Cancer Prev. 2021; 22(9): 2929-38.
14. Hasbiyani NA, Wulandari F, Nugroho EP, Hermawan A, Meiyanto E. Bioinformatics analysis confirms the target protein underlying mitotic catastrophe of 4T1 cells under combinatorial treatment of PGV-1 and galangin. Sci Pharm. 2021; 89(3): 38. doi: 10.3390/

scipharm89030038.

15. Bracci L, Schiavoni G, Sistigu A, Belardelli F. Immune-based mechanisms of cytotoxic chemotherapy: Implications for the design of novel and rationale-based combined treatments against cancer. Cell Death Differ. 2014; 21(1): 15-25.
16. Barrios CH. Global challenges in breast cancer detection and treatment. Breast. 2022; 62 Suppl 1(Suppl 1): S3-6.
17. Aresta G, Araújo T, Kwok S, Chennamsetty SS, Safwan M, Alex V, *et al*. BACH: Grand challenge on breast cancer histology images. Med Image Anal. 2019; 56: 122-39.
18. Han HS, Vikas P, Costa RLB, Jahan N, Taye A, Stringer-Reasor EM. Early-stage triple-negative breast cancer journey: Beginning, end, and everything in between. Am Soc Clin Oncol Educ Book. 2023; 43: e390464. doi: 10.1200/EDBK_390464.
19. Onitilo AA, Engel JM, Greenlee RT, Mukesh BN. Breast cancer subtypes based on ER/PR and Her2 expression: Comparison of clinicopathologic features and survival. Clin Med Res. 2009; 7(1-2): 4-13.
20. Ghasemi M, Turnbull T, Sebastian S, Kempson I. The MTT assay: Utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. Int J Mol Sci. 2021; 22(23): 12827. doi: 10.3390/ijms222312827.
21. Freimoser FM, Jakob CA, Aebi M, Tuor U. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a fast and reliable method for colorimetric determination of fungal cell densities. Appl Environ Microbiol. 1999; 65(8): 3727-9.
22. Chou TC. Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer Res. 2010; 70(2): 440-6.
23. Meiyanto E, Husnaa U, Kastian RF, Putri H, Larasati YA, Khumaira A, *et al*. The target differences of anti-tumorigenesis potential of curcumin and its analogues against HER-2 positive and triple-negative breast cancer cells. Adv Pharm Bull. 2021; 11(1): 188-96.
24. Meiyanto E, Putri H, Larasati YA, Utomo RY, Jenie RI, Ikawati M, *et al*. Anti-proliferative and anti-metastatic potential of curcumin analogue, pentagamavunon-1 (PGV-1), toward highly metastatic breast cancer cells in correlation with ROS generation. Adv Pharm Bull. 2019; 9(3): 445-52.
25. Castedo M, Perfettini JL, Roumier T, Andreau K, Medema R, Kroemer G. Cell death by mitotic catastrophe: A molecular definition. Oncogene. 2004; 23(16): 2825-37.
26. Sazonova EV, Petrichuk SV, Kopeina GS, Zhivotovsky B. A link between mitotic defects and mitotic catastrophe: Detection and cell fate. Biol Direct. 2021; 16(1): 25. doi: 10.1186/s13062-021-00313-7.
27. Ogrodnik M. Cellular aging beyond cellular senescence: Markers of senescence prior to cell cycle arrest in vitro and in vivo. Aging Cell. 2021; 20(4): e13338. doi: 10.1111/acel.13338.
28. Al-Mahmood S, Sapiezynski J, Garbuzenko OB, Minko T. Metastatic and triple-negative breast cancer: Challenges and treatment options. Drug Deliv Transl Res. 2018; 8(5): 1483-507.
29. Gupta SC, Patchva S, Koh W, Aggarwal BB. Discovery of curcumin, a component of golden spice, and its miraculous biological activities. Clin Exp Pharmacol Physiol. 2012; 39(3): 283-99.
30. Troselj KG, Kujundzic RN. Curcumin in combined cancer therapy. Curr Pharm Des. 2014; 20(42): 6682-96.
31. Yu S, Kim T, Yoo KH, Kang K. The T47D cell line is an ideal experimental model to elucidate the progesterone-specific effects of a luminal A subtype of breast cancer. Biochem Biophys Res Commun. 2017; 486(3): 752-8.
32. Wei LL, Miner R. Evidence for the existence of a third progesterone receptor protein in human breast cancer cell line T47D. Cancer Res. 1994; 54(2): 340-3.