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# Hesperidin Enhanced the Antimigratory Activity and Senescence-Mediated G2/M Arrest Effect of PGV-1 Against T47D Luminal Breast Cancer Cells

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**ABSTRACT**

Luminal breast cancer cells exhibit proliferative and metastatic characteristics. This study aims to confirm the effect of the combination treatment of Pentagamavunone-1 (PGV-1), a potent anticancer candidate, and hesperidin on the luminal breast cancer cell line T47D covering the evaluation of cytotoxic and antimigratory activities of PGV-1 and analyzing the most probable protein targets of PGV-1 and hesperidin. Cytotoxic effects were assayed via trypan blue exclusion. Cell cycle profiles were analyzed through flow cytometry. Cellular senescence phenomena were observed through senescence-associated β-galactosidase assay. Cell migration was evaluated by performing a scratch wound healing assay. Molecular target prediction was conducted through bioinformatics analysis with UALCAN and molecular docking with Molecular Operating Environment (MOE) software. PGV-1 showed considerably stronger cytotoxicity ($IC_{50}$ value of 2 μM) than hesperidin ($IC_{50}$ value of 200 μM). However, the combination of PGV-1 and hesperidin exhibited a synergistic effect (combination index < 0.3) and increased the populations of cells at the G2/M phase and senescent cells. This effect might be correlated to its antiproliferative properties. In addition, the scratch wound healing assay showed that the combination treatment inhibited cell migration remarkably. Molecular docking demonstrated the potential interactions of PGV-1 and hesperidin with their protein targets, i.e., KIF1, CDK1, TOP2A, CA12, ESR1, FN1, and TYMS, in the cell cycle machinery. Altogether, the findings of this work strengthen the evidence showing that combining PGV-1 with hesperidin enhances the anticancer properties of PGV-1 against luminal breast cancer.

**Keywords:** curcumin analog PGV-1, hesperidin, co-treatment, cell cycle, cell migration

## INTRODUCTION

Pentagamavunone-1 (PGV-1, with the IUPAC name of 2,5-bis-[4-hydroxy, 3',5'-dimethyl]-benzylidine-cyclopentanone) is a synthetic compound with high potential for development as a new anticancer agent. In addition to showing strong cytotoxic properties against several cancer cells, this curcumin analog is effective in inhibiting tumor growth *in vivo*. PGV-1 suppresses the proliferation of various types of cancer cells, including breast cancer cells (Meiyanto *et al*. 2019), colon cancer cells (Wulandari *et al*. 2021), and leukemia cells (Lestari *et al*. 2019), with $IC_{50}$ values of 0.1–6 μM. Nevertheless, the cytotoxicity values of PGV-1 generally indicate that the cytostatic potential of PGV-1 remains below that of commercial cytotoxic agents, such as doxorubicin and paclitaxel. However, PGV-1 shows better selectivity than other cytotoxic agents because it does not exert a remarkable cytotoxic effect on

noncancerous cells (Wulandari *et al*. 2021). In mouse models, PGV-1 also suppress the growth of breast tumors and colorectal tumors when orally administered (Lestari *et al*. 2019; Wulandari, 2023). Moreover, in mice, PGV-1 does not show a considerable toxic effect on general body condition, such as blood profiles and body weights (Wulandari, 2023). Therefore, developing PGV-1 continuously as an anticancer agent by increasing its cytostatic potential is interesting.

PGV-1 exerts a synergistic cytotoxic effect with the chemotherapeutic agent doxorubicin or 5-fluorouracil (5-FU) against several cancer cell lines, such as MCF-7 (Meiyanto *et al*. 2014), 4T1 (Meiyanto *et al*. 2019), T47D (Da'i *et al*., 2012), and WiDr (Meiyanto *et al*. 2018; Wulandari *et al*. 2021) because it has specific targets in the cell cycle during the progression of the G2/M phase (Da'i *et al*., 2012; Lestari *et al*. 2019). Given these different targets of action, PGV-1 makes an important contribution to amplifying cytotoxic effects on cancer cells when it is combined with doxorubicin or 5-FU. However, doxorubicin and 5-FU are not selective to normal cells and have side effects, such as kidney, heart, and intestinal damage (Meiyanto *et al*. 2019). Therefore, PGV-1 can be developed by combining it with agents that are safe but still provide a synergistic effect against cancer cells.

The promising potential of PGV-1 as a chemotherapeutic agent opens opportunities for development by increasing the effectiveness of its anticancer activity while retaining its low side effects. Previous studies have shown that in 4T1 cells, the combination of PGV-1 with potential natural compounds, such as galangin, piperine, and diosmin, has a synergistic effect in subduing proliferation through the induction of senescence and mitotic catastrophe (Endah *et al*. 2022; Hasbiyani *et al*. 2021; Musyayyadah *et al*. 2021). In the present study, we used the potential natural ingredient compound hesperidin, an unsaturated flavonoid glycoside that is commonly found in orange peels (Ikawati *et al*. 2019; Meneguzzo *et al*. 2020; Putri *et al*. 2022). Hesperidin inhibits the cell cycle; induces cellular senescence and apoptotic cell death; and promotes oxidative stress in cancer cells, including the triple negative breast cancer cell line MDA-MB-231, luminal breast cancer cell line MCF-7, T47D, and SK-BR-3, hepatocellular carcinoma cell line HepG2, and cervical adenocarcinoma cell line HeLa (Ferreira de Oliveira *et al*. 2020). Hesperidin has been demonstrated to inhibit cell proliferation in several cancer cell models by elevating p53, cyclin-dependent kinase inhibitors, and caspase 3 activation or inhibiting the Akt signaling pathway, NF-kB, and matrix metalloproteinase (MMP)-9 and MMP-2 activity (Ferreira de Oliveira *et al*. 2020; Kongtawelert *et al*. 2020). Moreover, hesperidin is not toxic to normal NIH-3T3 fibroblast cells and normal Vero kidney cells (data not shown). The safety of hesperidin can be demonstrated by its common use as a hemorrhoid or venaroid drug and as a health supplement (Nagasako-Akazome, 2014; Zanwar *et al*. 2014).

The above findings indicate that hesperidin with its target mechanism in the cell cycle machinery and signal transduction will provide a potential synergistic effect with PGV-1 that of more specific targets on mitotic arrest. In this study, we applied the combination of PGV-1 with hesperidin against estrogen receptor-positive ($ER^+$) or luminal breast cancer, a type of cancer with high mortality rates (Kulkarni *et al*. 2019). Although the estrogen receptor-targeted therapy of luminal breast cancer is already developed, its outcomes remain unsatisfactory due to increasing toxicity and resistance. PGV-1 promises to challenge this situation because it exhibits a strong cytotoxic effect on T47D cells, the representative luminal breast cancer cell line (Da'i, 2012). Therefore, we used the T47D cell line as a model to evaluate the cytotoxicity and physiological effects of the combination of PGV-1 and hesperidin. The T47D cancer cell line is progressive, metastatic, and highly motile due to the mutation of *TP53* (Abuhamad *et al*. 2022). Therefore, the present work is interesting as a preliminary study on the application of the combination of PGV-1 and hesperidin as a treatment for metastatic $ER^+$ breast cancer. In our study, we observed the cytotoxic effects, cell cycle arrest, senescence, and cell migration of T47D breast cancer cells under PGV-1 and hesperidin treatments alone and in combination. In addition, we utilized bioinformatics approaches to provide supporting information regarding the target proteins that are responsible for cell cycle arrest in $ER^+$ breast cancer and to determine the inhibitory potential of hesperidin and PGV-1 compounds.

## MATERIALS AND METHODS

### Ethical approval

All experiments were approved by the Medical and Health Ethical Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, No. KE/FK1012/EC/2020.

**Cell culture**

The T47D cell line was provided by Professor Masashi Kawaichi, Nara Institute of Science and Technology, Japan. The cells were grown in Roswell Park Memorial Institute (RPMI) medium added with 10% v/v fetal bovine serum (Sigma) and 150 IU/mL penicillin–150 µg/mL streptomycin (Gibco).

**Compounds**

Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, synthesized PGV-1 with 95% purity. Hesperidin with 95% purity was bought from Sigma Chemical Co. (St. Louis, MO, the USA). PGV-1 and hesperidin stock solutions were prepared by dissolving each compound in dimethyl sulfoxide (Sigma) to a maximum final concentration of 0.1 µM. Subsequently, each stock solution was serially diluted with RPMI to a certain range of concentrations.

**Cytotoxicity assay**

T47D cells were seeded into a 24-well plate at a density of $5 \times 10^4$ cells/mL per well and incubated for 24 h. The cells were then treated with various concentrations of PGV-1, hesperidin, and their combination for 24 h. The treated cells were washed with phosphate-buffered saline (PBS) and subjected to trypan blue exclusion assay to count viable cells as previously described (Musyayyadah *et al*., 2021).

**Cell cycle analysis**

T47D cells were seeded into a 6-well plate at a density of $2 \times 10^5$ cells/mL per well and incubated for 24 h. Next, the cells were treated for 24, 48, and 72 h with PGV-1, hesperidin, or a combination of the two drugs. After incubation, all cells were collected by using 0.25% trypsin–EDTA (Sigma). After the cells were washed with PBS, they were fixed in 500 µL of 70% ethanol and stained by using a propidium iodide (PI) (Sigma) staining solution containing RNAse and Triton-X 100. The cells were incubated for five min at room temperature and then transferred to a flow cytometer tube. A BD FACSDiva 8.0.2 flow cytometer was used to measure cell distribution.

**Senescence-associated β-galactosidase assay**

T47D cells were seeded into a 6-well plate at a density of $2 \times 10^5$ cells/mL per well and incubated for 24 h. The cells were then treated with different concentrations of PGV-1, hesperidin, and their combination for 24 h. They were washed with PBS and fixed by adding 4% paraformaldehyde and 0.2% glutaraldehyde for approximately 20 min. The cells were washed with PBS and then stained by using a mixed X-gal solution (1 mg/mL X-gal, 5 mM $K_4Fe[CN]_6$, 5 mM $K_3Fe[CN]_6$, PBS 2× [pH 6.0], and 2 mM $MgCl_2$). After 72 h of incubation, stained cells were carefully observed under an inverted microscope at 200× magnification. Green cells were representative of β-galactosidase-positive cells and quantified with ImageJ.

**Scratch wound healing assay**

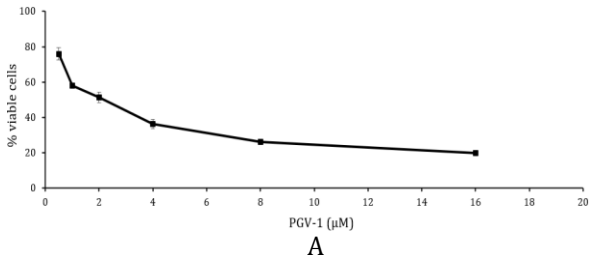
The cells were grown and incubated for 24 h in a 24-well plate at a density of $8 \times 10^4$ cells per well. They were starved with serum-free medium containing 1 µg/mL mitomycin C solution (Nacalai Tesque) for 18 h. The cells were washed with PBS and scratched with a sterile yellow pipette tip. They were treated with PGV-1, hesperidin, and their combination. Wound closures were documented at 0, 18, 24, and 42 h and quantified with ImageJ software.

**Protein target prediction analysis**

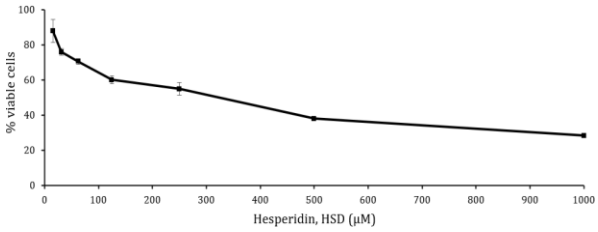
The protein targets of PGV-1 and hesperidin were predicted by utilizing SwissTargetPrediction (http://www.swisstargetprediction.ch/). Gene expression levels in luminal breast cancer and normal tissues were examined by using the UALCAN database (http://ualcan.path.uab.edu). By applying the www.interactivenn.net/ website, the protein targets were then compared with the genes overexpressed in luminal breast cancer.

**Virtual molecular interaction analysis**

The characteristics of the molecular binding of PGV-1 and hesperidin with their protein targets were analyzed by using the licensed software Molecular Operating Environment (MOE) 2010.10 (license of Faculty of Pharmacy, Universitas Gadjah Mada). This software was applied to calculate root mean standard deviations (RMSDs) and docking scores to determine the properties of the binding between ligands and proteins. Triangle matcher and London ΔG were used for placement settings and scoring. The force field technique was applied to filter the docking outcomes from 10 retained settings. The structures of hesperidin and PGV-1 were visualized by using a chemical builder in MOE and then prepared for structural energy minimization and conformational structure production in MOE. This assay was conducted at the binding site on each protein's native ligand.




A

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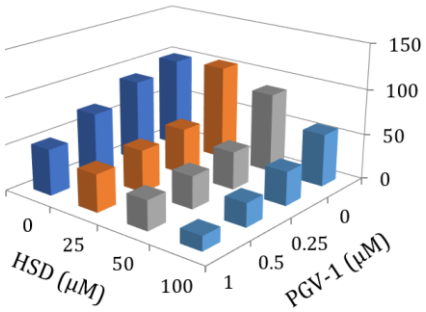


C

| Compound | $IC_{50}$ (µM) |
|---|---|
| PGV-1 | 2 |
| Hesperidin | 200 |

D

| Combination Index (CI) | | | | |
|---|---|---|---|---|
| | | PGV-1 (µM) | | |
| | | 0.25 | 0.5 | 1 |
| Hesperidin (µM) | 25 | 0.2 | 0.2 | 0.2 |
| | 50 | 0.2 | 0.2 | 0.2 |
| | 100 | 0.2 | 0.2 | 0.2 |

E

Figure 1. Analysis of the cytotoxicity of PGV-1 and hesperidin against T47D cells *in vitro*. Cytotoxic activity of (A) PGV-1, (B) hesperidin, and (C) combination of PGV-1 and hesperidin against T47D cells. Cell viability was evaluated with the trypan blue exclusion assay. $IC_{50}$ (D) and CI (E) of PGV-1 and hesperidin for T47D cells. Results are the average of three independent experiments (mean ± SD).

The docking scores and visualizations of the binding of each drug to its protein targets were used to predict affinity.

**Data analysis**

All data were calculated in the form of means to obtain standard deviations or standard errors. One-way ANOVA and Tukey post hoc analysis were performed (SPSS version 21, Chicago, the USA) to analyze the significance and variance of each treatment. $p < 0.05$ was considered significant. Synergistic effects were determined on the basis of the Chou and Talalay formula (Zulfin *et al*. 2021).

## RESULTS AND DISCUSSION

### Cytotoxic activity of PGV-1 and hesperidin in luminal breast cancer

T47D cells were used as a luminal breast cancer cell model and subjected to trypan blue exclusion assay (Jenie et al. 2020) to observe the cytotoxic activities of PGV-1 and hesperidin. The cells were treated for 24 h with PGV-1 (0.5–16 µM) or hesperidin (10–1000 µM) (Figures 1A–B). Cytotoxic activity is expressed in $IC_{50}$, the concentration that inhibits the viability of 50% of cells. PGV-1 showed a strong cytotoxic effect ($IC_{50}$ of 2 µM), whereas hesperidin had a relatively weak cytotoxic effect ($IC_{50}$ of 200 µM) (Figure 1D). The cytotoxic activity of the combination treatment was calculated and presented in the form of a combination index (CI) (Chou 2010) (Figure 1C). The results showed that at all tested concentrations, the combination of PGV-1 with hesperidin decreased the viability of T47D cells significantly with CI <0.3, indicating that cotreatment with both compounds exhibited synergistic properties (Figure 1E). The causative mechanism of this synergistic effect requires further investigation.

### PGV-1 in combination with hesperidin regulates the T47D cell cycle

We performed cell cycle analysis by using PI staining with flow cytometry to investigate the synergistic cytotoxicity of PGV-1 and hesperidin further. This assay showed that treatment with PGV-1 alone at both concentrations significantly increased the accumulation of the cell population at the G2/M phase (Figure 2). We also observed a similar effect under treatment with hesperidin alone. Specifically, hesperidin alone also caused an increase in G2/M cell cycle arrest.

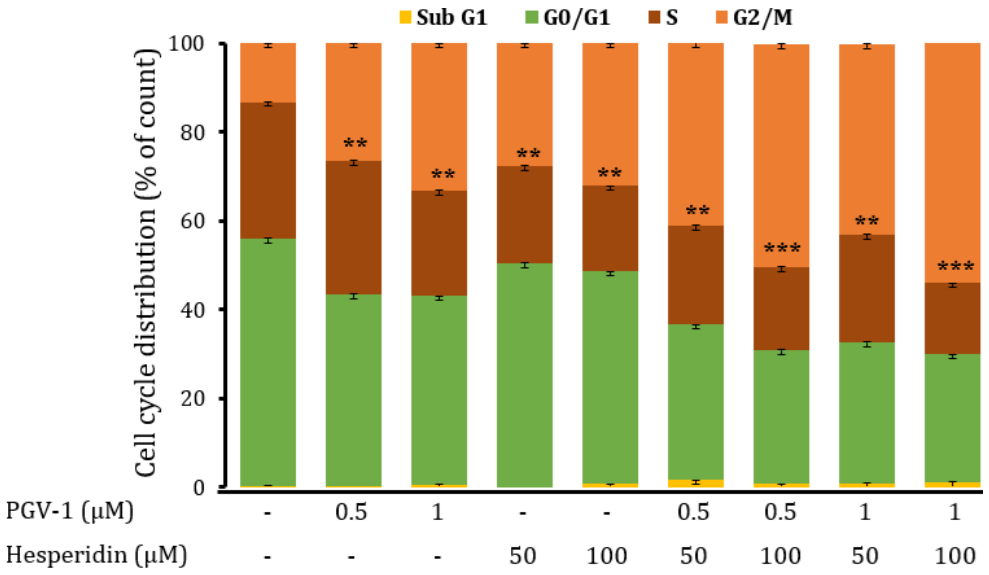


Figure 2. Regulatory effect of the combination of PGV-1 and hesperidin on the T47D cell cycle. T47D cells ($2 \times 10^5$ cells/well) were treated with PGV-1, hesperidin, and their combination for 24 h and then stained with PI. Cell accumulation was analyzed by using flow cytometry with $^{**}p < 0.01$ and $^{***}p < 0.001$.

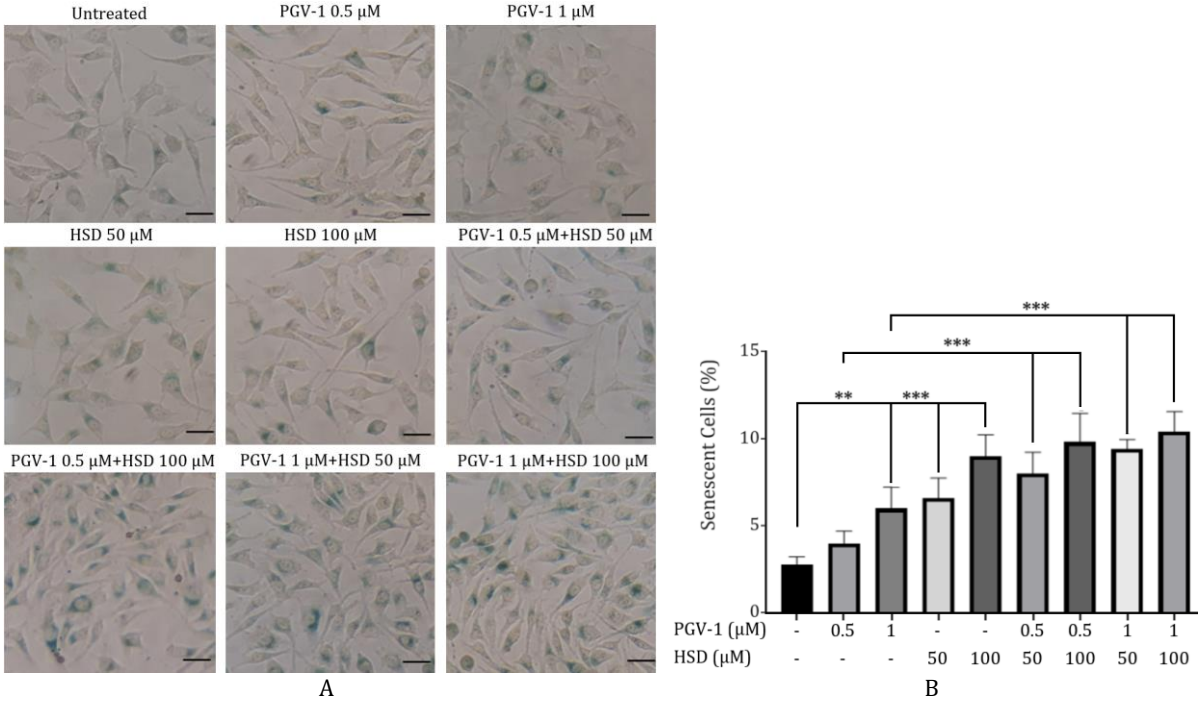


Figure 3. Evidence of T47D cell senescence after treatment with PGV-1, hesperidin, and their combination. T47D cells ($2 \times 10^5$ cells/well) were given PGV-1, hesperidin, and their combination for 24 h before being stained with β-galactosidase. (A) Cell morphology after 72 h of staining viewed under an inverted microscope at 200× magnification. (B) Number of senescent cells (%) statistically analyzed through one-way ANOVA. $^{**}p < 0.01$ and $^{***}p < 0.001$.

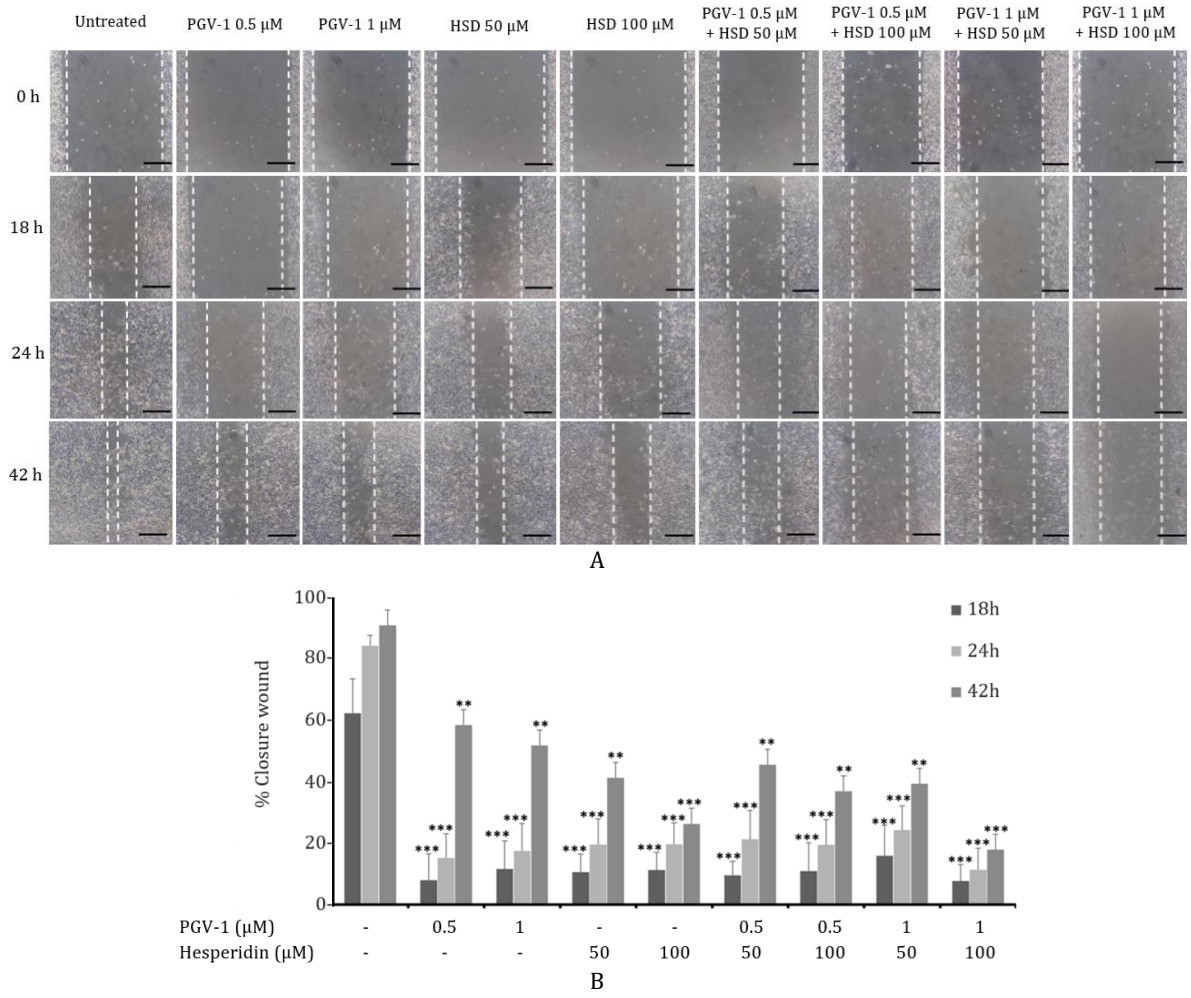


Figure 4. Inhibitory effects of PGV-1, hesperidin, and their combination on T47D cell migration. The effect of both compounds on T47D cell migration was evaluated through a scratch wound healing assay. T47D cells were first scratched and treated with PGV-1 and hesperidin alone and in combination. (A) Morphology of T47D cells after scratching and treatment for 0, 18, 24, and 48 h viewed under an inverted microscope at 100× magnification. (B) Percentage of T47D cell closure after treatments. The area of closure was calculated by using ImageJ software (n = 3) with **p < 0.01 and ***p < 0.001.

Meanwhile, the combination of PGV-1 and hesperidin at all concentrations caused G2/M phase arrest and increased the accumulation of cells at the sub-G1 phase. The accumulation of cells at the sub-G1 phase might be a manifestation of cell apoptosis (Wulandari *et al*. 2021). This result proved that the synergistic effect of the two compounds may be due to the induction of G2/M cell cycle arrest and possibly apoptosis. Cell cycle arrest that leads to apoptosis is generally mediated by senescence events and is permanent due to irreversible intracellular damage (Larasati *et al*. 2018). Hence, an assay must be conducted to examine the effect of PGV-1 and hesperidin on the senescence of T47D cells.

**Effect of PGV-1 and hesperidin on T47D cellular senescence**

We used the SA-β-galactosidase (SA-β-gal) assay to determine the relationship between cell cycle arrest phenomena and cellular senescence and the influence of PGV-1, hesperidin, and their combination on cellular senescence to further our understanding of the close correlation between cell cycle arrest and cellular senescence.

The number of senescent cells is represented by the intensity of the green color in senescent cells (Zulfin *et al*. 2021). The concentrations used in the senescence assay were set in reference to the obtained $IC_{50}$ values of the single or combination treatment. Hence, the concentrations of PGV-1 used in this assay were 0.5 and 1 μM, whereas those of hesperidin were 50 and 100 μM, which are a quarter and a half of the $IC_{50}$ values of the single or combination treatments, respectively. Green T47D cells were SA-β-gal-positive cells representing senescent cells (Figure 3A). Treatment with 1 μM PGV-1 alone significantly increased senescent cells ($p < 0.01$). Meanwhile, treatment with 50 and 100 μM hesperidin alone increased senescent cells significantly ($p < 0.001$). Compared with that under single treatment, the number of senescent cells increased significantly under combined treatment with PGV-1 and hesperidin at both concentrations ($p < 0.001$) (Figure 3B). This piece of evidence indicated that PGV-1 and hesperidin, whether alone or in combination, can induce cell senescence, and supported that the inhibition of cell proliferation causes cell cycle arrest and cellular senescence.

**Effect of PGV-1 and hesperidin on cell migration**

As previously mentioned, metastatic ability is a characteristic of luminal breast cancer (Li *et al*. 2013; Negro *et al*. 2020). Therefore, we conducted a migration study on T47D cells to investigate their migratory properties preliminarily. Previously, we found that PGV-1 inhibits the migration of 4T1, a triple negative breast cancer cell line, at the concentration of 4 μM (Meiyanto *et al*. 2019). In this experiment, we combined PGV-1 with hesperidin to discover if hesperidin can increase the antimigratory effect of PGV-1 on T47D breast cancer cells. A scratch wound healing assay was performed with low concentrations to avoid introducing cytotoxicity bias to cell migration properties. Cell morphology observation showed that the wound in the untreated group had almost completely closed at 42h (Figure 4A). The antimigratory effect of PGV-1 in combination with hesperidin seemed to have increased relative to that of PGV-1 alone. In particular, 0.5 μM PGV-1 in combination with 50 μM hesperidin, 0.5 μM PGV-1 in combination with 100 μM hesperidin, and 1 μM PGV-1 in combination with 100 μM hesperidin ($p < 0.05$) reduced T47D cell migration at 42 h (Figure 4B). Additional experimental studies must be conducted given that PGV-1 and hesperidin may target different proteins involved in cell migration.

**Molecular interaction of PGV-1 and hesperidin with their protein targets**

Through bioinformatics analysis, we identified the interesting specific protein targets of each compound that are overexpressed in luminal breast cancer. These targets were KIF11, CDK1, and TOP2A for PGV-1 and CA12, ESR1, FN1, and TYMS for hesperidin (Supplementary Figure). We used MOE version 10.1 to run a molecular docking simulation to confirm these specific targets. We applied Filanesib, 4A2, Etoposide, 84I, 17-BET, 4-OHT, NAG, and MTX as the native ligands of KIF11, CDK1, TOP2A, CA12, ESR1, FN1, and TYMS, respectively. The structures of the predicted target proteins were downloaded via PDB database (http://rcsb.org/pdb/) in .pdb file format with the following PDB IDs: KIF11 (6ZHW), CDK1 (3ZCW), TOP2A (6ZY6), CA12 (7PUW), ESR1 (5FQR), FN1 (3M7P), and TYMS (7DP4). We discovered that all compounds showed less or the same binding energy on the respective proteins as the respective native ligands after validation with RMSD values less than 2 A, except for PGV-1 with protein target KIF11, which showed more binding energy compared to its native ligand (Table I). We analyzed the agonist and antagonist sites of the ESR1 protein. Hesperidin can bind at both sites with considerably lower binding energy than native ligands. This result might show that hesperidin could inhibit the activity of ESR1 (Figure 5). Both compounds also showed almost the same amino acid residues as the native ligands of their protein targets. Therefore, PGV-1 might be able to inhibit the activities of KIF11 and CDK1. Meanwhile, in accordance with its docking scores, hesperidin could inhibit CA12, ESR1, FN1, and TYMS proteins.

The objective of this research is to evaluate further the potential of PGV-1 combined with hesperidin as an anticancer agent against the luminal breast cancer cell line T47D on the basis of their cytotoxic and physiological effects. Through an in vitro cytotoxicity assay, we found that PGV-1 and hesperidin had $IC_{50}$ values of 2 and 200 μM for T47D cells, showing strong and low cytotoxicity, respectively (Prayong *et al*. 2008). PGV-1 exhibits cytotoxic activity against various cancer cells, such as 4T1, K562, and WiDr cells (Lestari *et al*. 2019; Meiyanto *et al*. 2021; 2019; 2018; Wulandari *et al*. 2021). Meanwhile, hesperidin possesses low cytotoxicity against macrophage cells (Ikawati *et al*. 2020).

Table I. Comparison of the molecular docking results of PGV-1 and hesperidin

| Protein name | Small molecule | Docking score | RMSD | Amino acid residue |
|---|---|---|---|---|
| KIF11 (6GU6) | Dinaciclib | −5.2385 | 0.8267 | Lys A89, Lys A33, Phe A80 |
| | PGV-1 | −6.6131 | 1.7042 | Lys A89, Ile A10 |
| CDK1 (3ZCW) | Native ligand (4A2) | −7.0216 | 1.1821 | Glu 116, Lys 216 |
| | PGV-1 | −9.6501 | 0.1632 | Asn 18, Thr 300, |
| TOP2A (6ZY6) | Etoposide | −4.2923 | 1.2540 | DC B1, DG C13 |
| | PGV-1 | −2.6283 | 1.0663 | - |
| CA12 (7PUW) | Native ligand (84I) | −11.0987 | 0.8675 | Thr 198, Thr 199, ZN 301, Val 119, His 91, Asn 64 |
| | Hesperidin | −14.1864 | 1.6640 | Glu 254, Arg 194 |
| ESR1 (1ERE | 17-BET (agonist) | −11.9375 | 0.2970 | His 524, Glu 353 |
| agonist site) | Hesperidin | −16.2881 | 1.4046 | Glu 353, Trp 383, Ile 386, Ile 452, Leu 511 |
| ESR1 (3ERT | 4-OHT (antagonist) | −14.2179 | 1.0201 | Glu 353, Arg 394, Leu 387, Ile 424 |
| antagonist site) | Hesperidin | −17.3415 | 1.7546 | Ile 452, Ile 514 |
| FN1 (3M7P) | Native ligand (NAG) | −4.8941 | 1.6528 | Gln 450, His 421, Pro 423, Asn 430 |
| | Hesperidin | −12.7384 | 1.2990 | Cys 374, Thr 376, Thr 375, Thr 325 |
| TYMS (7DP4) | Methotrexate (MTX) | −15.8985 | 1.7091 | Met 302, Ile 101, Asp 211 |
| | Hesperidin | −18.9747 | 1.9620 | Phe 218, Trp 102, Tyr 73, Gly 76, V 77, Glu 79, Lys 75, Tyr 286, Trp 74 |

In this study, the combination of PGV-1 and hesperidin presented synergistic effects in reducing the viability of T47D cells. This result demonstrated that hesperidin enhanced the cytotoxic activity of PGV-1 possibly through a distinctive mechanism. Another possibility is that hesperidin can maintain the accumulation of PGV-1 in cells by inhibiting cellular efflux, as observed in MCF-7 cells (Sarmoko *et al*. 2014). At the same time, both compounds were thought to be safe for normal cells. The stronger cytotoxic effect of PGV-1 than that of hesperidin may be due to the targets of PGV-1 in the central machinery of cell division (Lestari *et al*. 2019).

For the further evaluation of the anticancer activity of PGV-1 and hesperidin, we observed their ability to modulate cell cycle progression. Treatment with PGV-1 or hesperidin alone induced cell cycle arrest in the G2/M phase. The cytotoxicity assay results revealed that PGV-1 in combination with hesperidin could also promote cell cycle arrest at the G2/M phase. Moreover, both treatments induced sub-G1 accumulation, which possibly leads to apoptosis, but still in a small portion. Therefore, the incidence of cell cycle arrest at the G2/M phase is likely to be related to the occurrence of cell senescence. This phenomenon was also found in K562 cells, which exhibited mitotic arrest that led to senescence (Endah *et al*. 2022). Similar to previous studies (Meiyanto *et al*. 2021; 2019), the present study confirmed that PGV-1 inhibited the migration of T47D cells. Hesperidin enhanced this effect, indicating that it not only increased the cytotoxic effect of PGV-1 through cell cycle modulation but also played a role in disrupting metastatic processes. However, comparison with other G2/M phase-targeting compounds, such as taxol or nocodazol, remains challenging.

We predicted the protein targets of PGV-1 and hesperidin through bioinformatics studies, which identified seven different proteins. In luminal breast cancer, PGV-1 mainly targeted catalytic enzymes, such as KIF11, CDK1, and TOP2A. Meanwhile, hesperidin appeared to target cell proliferation and differentiation enzymes, such as CA12, ESR1, FN1, and TYMS. In general, the protein targets of PGV-1 and hesperidin have distinct roles in cell physiological processes. The protein targets of PGV-1 function as catalytic enzymes in cell cycle activities, such as mitotic spindle formation and DNA synthesis. CDK1, a protein target, plays a key function in the control of the G2/M phase (Izadi *et al*. 2020). ESR1, hesperidin's protein target, is a transcription factor that regulates the cell cycle (Brett *et al*., 2021).

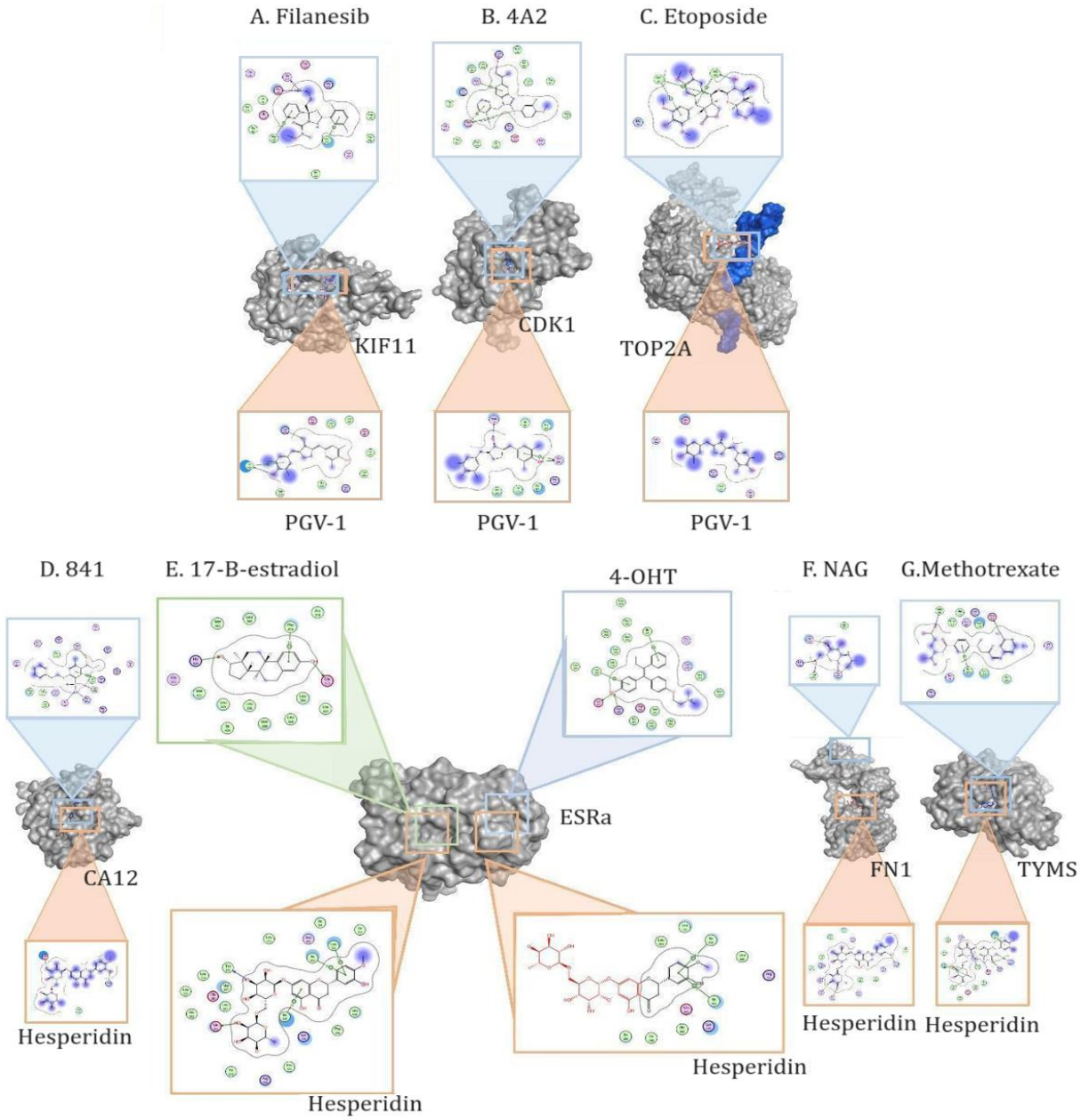


Figure 5. Analysis of the molecular docking of PGV-1 and hesperidin with KIF11, CDK1, TOP2A, CA12, ESR1, FN1, and TYMS compared with their native ligands. Docking performances of (A) PGV-1 to KIF11, (B) PGV-1 to CDK1, (C) PGV-1 to TOP2A, (D) hesperidin to CA12, (E) hesperidin to ESR1, (F) hesperidin to FN1, and (G) hesperidin to TYMS.

These protein targets are thought to be important for the anticancer activities of PGV-1 and hesperidin in luminal breast cancer (Supplementary Table). Future studies may need to compare the cell growth inhibitory activities of PGV-1 and hesperidin with those of ER inhibitors, such as tamoxifen or 4-hydroxy tamoxifen.

In addition, we performed molecular docking to verify further the potential interactions of PGV-1 and hesperidin with their protein targets. The molecular docking results indicated that PGV-1 and hesperidin bound more strongly to the protein targets than the native ligands. In particular, hesperidin could bind to the

agonist and antagonist sites of its protein target ESR1, indicating that it might inhibit the activity of the ESR1 protein. Meanwhile, PGV-1 showed its lowest binding energy with its protein target CDK1, confirming that its main mechanism of action is its potential to target mitosis. This phenomenon suggests that both compounds have the potential to inhibit the activity of their protein targets.

Overall, the combination of PGV-1 and hesperidin targeted several distinct proteins that may be correlated with cell cycle arrest in the G2/M phase and cellular senescence induction. In addition, these protein targets might be involved in the antimigratory properties of the combination of PGV-1 and hesperidin. These findings provide interesting insights into the potential of PGV-1 and hesperidin as anticancer agents. However, this research remains limited to partial physiological and mechanistic studies on PGV-1 and hesperidin. Therefore, further experiments to deepen our understanding of both compounds' anticancer properties are interesting challenges.

## CONCLUSION

The combination of PGV-1 and hesperidin had synergistic effects in the inhibition of T47D cell proliferation. Furthermore, the combination of both compounds resulted in G2/M cell cycle arrest and cellular senescence and presented antimigratory properties. The combination of PGV-1 and hesperidin targeted several distinct proteins. Specifically, PGV-1 targeted KIF11, CDK1, and TOP2A, whereas hesperidin targeted CA12, ESR1, FN1, and TYMS. Our interesting findings elucidate the potential of PGV-1 and hesperidin as anticancer agents.

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## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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