

Cytotoxic Activity and Senescence Modulatory Effect of Hesperetin on Triple-Negative Breast Cancer Cells and Kidney Cells Co-Treatment with Cisplatin

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Abstract

Cisplatin (Cisp) is a non-specific chemotherapeutic agent for breast cancer. Hesperetin (HST), a flavanone found in various citrus fruits, exhibits bioactive properties, functioning as an antioxidant, anti-inflammatory, and anticancer agent. The objective of this research was to investigate the potential of HST as a co-chemotherapeutic agent in conjunction with Cisp, specifically focusing on its cytotoxic effects against 4T1 triple-negative breast cancer cells and senescence modulatory effect on Vero normal kidney cells. The cytotoxic effect and viability cell of HST were evaluated through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. In addition, the effect of cellular senescence inhibition on the Vero cell line was measured using senescence-associated β -galactosidase (SA- β -gal) staining. In the MTT assay, both HST and cisplatin demonstrated a reduction in the viability of 4T1 cells in a dose-dependent manner, yielding IC_{50} values of 498 μ M and 2 μ M, respectively. The co-treatment of HST and cisplatin showed an increase in sensitivity of the 4T1 cells with a combination index of <1 . HST showed low cytotoxic activity against Vero cells, with IC_{50} values of over 500 μ M. HST decreased cellular senescence induced by cisplatin exposure on Vero cells. These results indicated that HST in co-treatment with cisplatin decreased 4T1 cell viability synergistically. HST independently reduces the cellular senescence of normal cells. Consequently, HST holds promise for potential development as a co-treatment agent in combination with cisplatin for breast cancer cells, and it may also serve as an alternative for counteracting senescence in healthy tissues.

Keywords: cytotoxic, senescence, hesperetin, cisplatin, breast cancer.

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INTRODUCTION

Cancer metastasis is the main cause of death in breast cancer. The mortality rate associated with breast cancer is attributed to the limited selectivity of antitumor agent therapy and the occurrence of metastasis and recurrence in individuals diagnosed with breast cancer (Du, *et al.*, 2010). Globally, breast cancer is the type of cancer with new cases and causes the highest number of deaths among all types of cancer in women. In several types of breast cancer, triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer and causes a poor prognosis in cancer patients. Several types of chemotherapy agents are used to inhibit the growth of breast cancer cells. Nevertheless, the efficacy of chemotherapy in treating triple-negative breast cancer (TNBC) is constrained by the absence of specific therapeutic molecular targets in this subtype (Mohamad, *et al.*, 2019).

Cisplatin (Cisp) is a non-specific chemotherapeutic agent for TNBC (Wahba, *et al.*, 2015). The mechanism of cisplatin as an anticancer agent is by interacting chemically directly with the N-7 position of guanine or adenine, resulting in covalent bonds within and between DNA strands (Zwelling, *et al.*, 1981). In particular, the use of Cisp is preferred because Cisp can be eliminated via the kidneys, providing limited side effects especially side effects related to the kidneys, nerves, and blood (Zhang, *et al.*, 2017). However, similar to other anticancer drugs, cisplatin also harms normal cells and cancer cells because it causes toxicity to organs outside the expected target, thus providing non-selective therapy (Pan, *et al.*, 2015). The use of chemotherapy drugs has side effects that result in cellular damage which leads to abnormalities and even damage to the kidney organs. Continuous exposure to agents that damage the kidneys will cause chronic kidney failure. This is characterized by damage to kidney cells which triggers a decrease in kidney function (GFR <60 mL/min/1.73m). One of the causes of kidney damage is an increase in reactive oxygen species (ROS) levels which triggers

oxidative stress which can worsen the physiological function of the kidneys. In this case, cisplatin is a chemotherapy agent that works by increasing ROS levels. Therefore, it is important to explore new agents that can regulate the development of TNBC breast cancer to reduce the mortality rate associated with this condition.

Flavonoids are prevalent in fruits, vegetables, and grains, with many Citrus species containing significant quantities of limonoids, flavonoids, and carotenoids in both glycoside and aglycone forms. Of the various types of flavonoids, naringenin and hesperetin (HST) are aglycone forms with the most significant flavanone groups (Yap, *et al.*, 2021). HST exhibits a range of pharmacological properties, including antioxidant effects (Khan, *et al.*, 2020), aromatase inhibition (Rahideh, *et al.*, 2017), and cytotoxicity against MCF-7 Her2 cells (Nurhayati, *et al.*, 2020), 4T1 cells (Yunita, *et al.*, 2020), and co-treatment with doxorubicin on MCF-7 Cells (Sarmoko, *et al.*, 2014). Some breast cancer chemotherapy is given together with other drugs. HST has also been shown to increase the effectiveness of chemotherapy such as that mediated by doxorubicin and cisplatin, on various cancer cells. However, a study HST combined with Cisp on TNBC breast cancer has not yet been conducted. Combination treatments using 2 or more different drugs have gained interest in the fight against cancer. Based on these studies, HST appears to enhance the response of 4T1 cells to cisplatin. The objective of this study was to investigate the potential use of HST as a co-treatment with cisplatin, with a particular focus on its cytotoxic impact on 4T1 cancer cells and its ability to modulate senescence in normal kidney cells.

MATERIALS AND METHODS

Cell culture

4T1 (ATCC® CRL-2539) breast cancer cells and Vero (ATCC® CCL-81) normal kidney cells were acquired from Cancer Chemoprevention

Research Center (CCRC), Yogyakarta, Indonesia. The cells were grown in DMEM medium containing 10% FBS (Sigma, St. Louis, Missouri, USA), 1.5% Penicillin-Streptomycin (Sigma), 0.5% Fungizone (Sigma), maintained at 37°C in an atmosphere of 5% CO₂. Cells were subcultured by employing trypsin-EDTA (Gibco, Invitrogen, USA) to facilitate their detachment. The substances utilized in the study were Hesperetin (Sigma) and Cisplatin (Wako, Japan).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) Cytotoxicity Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay was employed to evaluate cytotoxicity. Initially, a total of 8×10^3 4T1 cells were seeded into each well of a 96-well plate. The following day, the cells were exposed to varying concentrations of HST and Cisp for a duration of 24 h. Subsequently, 100 µL of 0.5 mg/mL MTT reagent (Biovision) was added to the cells and left to incubate for 2-4 h. Following the incubation period, a stopper solution containing SDS and 0.01N HCl was added to each well. The absorbance at 595 nm was subsequently measured using an ELISA reader, and the cell viability percentage was calculated based on the absorbance data. The IC₅₀ value was established by used linear regression analysis to correlate the sample concentration with cell viability percentage. The assessment of cytotoxic combinations involved evaluating the combination index using the method proposed by Reynold and Maurer in 2005.

Senescence-associated β-galactosidase (SA-βGal) Senescence-based Assay

The assessment of β-galactosidase expression associated with senescence was carried out using the SA-βGal assay. In this procedure, Vero cells (2×10^4 cells per well) were initially seeded in a 24-well plate and incubated for 24 h. Subsequently, the cells were washed twice with 1X

PBS. Afterward, a fixation buffer was introduced and allowed to stand for a specified period, followed by another wash with 1X PBS. An additional 650 µl of X-Gal solution was added, and the cells were then incubated at 37°C. After 72 h, the cells were observed under a microscope (Olympus CKX-41) at 200× magnification. The presence of blue cells indicated the presence of β-galactosidase-positive cells, indicative of senescent cells.

Data Analysis

The data were presented in the format of mean±SD and analyzed using SPSS 21.0 software. Oneway Anova was used to assess the statistical significance of differences between the untreated group and various treatment groups. The respective *p*-values (**p*<0.05; ***p*<0.01) have been provided within each figure in the experiments.

RESULTS

Cytotoxic Impact of Hesperetin (HST) on 4T1 and Vero Cells

The aim of this study was to investigate how HST affects the growth of TNBC breast cancer cells against normal kidney cells in *in vitro* conditions. Initially, we evaluated the cytotoxic impact of HST on both 4T1 and Vero cells. 4T1 cells were used as a representation of TNBC, while Vero cells were used as a model for kidney normal cells. HST was applied at concentrations ranging from 25 to 500 µM leading to a dose-dependent, $y = -0.1539x + 126.71$ with coefficient of correlation (R²)=R²=0.947. Cisp was applied at concentrations ranging from 0.1 to 10 µM leading to a dose-dependent, $y = -14.469x + 77.659$ with coefficient of correlation (R²)=R²=0.9798 (Figure 1A). The calculated IC₅₀ values for this effect were 498 µM, indicating that HST exhibited a moderate level of cytotoxicity (Prayong, *et al.*, 2008). Interestingly, when applied to concentrations up to 1000 µM, HST did not induce cytotoxicity in Vero cells with IC₅₀ value 577 µM (IC₅₀>500 µM) (Figure 1B.).

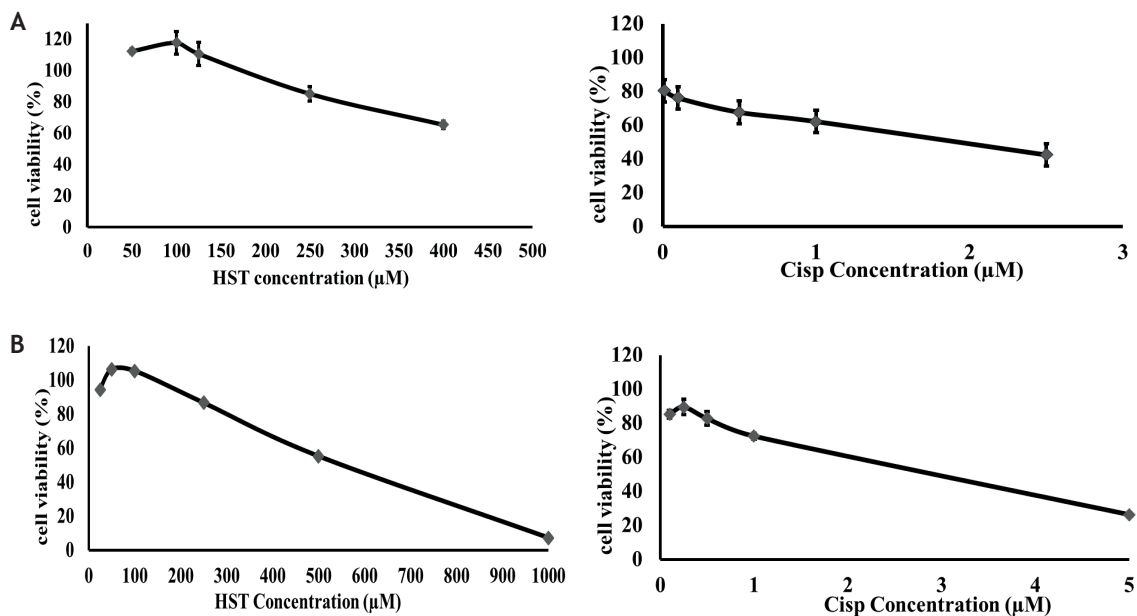


Figure 1. Cytotoxicity of hesperetin (HST) and cisplatin (Cisp) on 4T1 and Vero Cells. A. 4T1 cells (8×10^3 cells/mL) and B. Vero cells (1×10^4 cells/mL) were exposed to HST and Cisp for 24 h and subsequently measured using MTT assay. Data were means from three independent experiment $\pm$ standard error (SE).

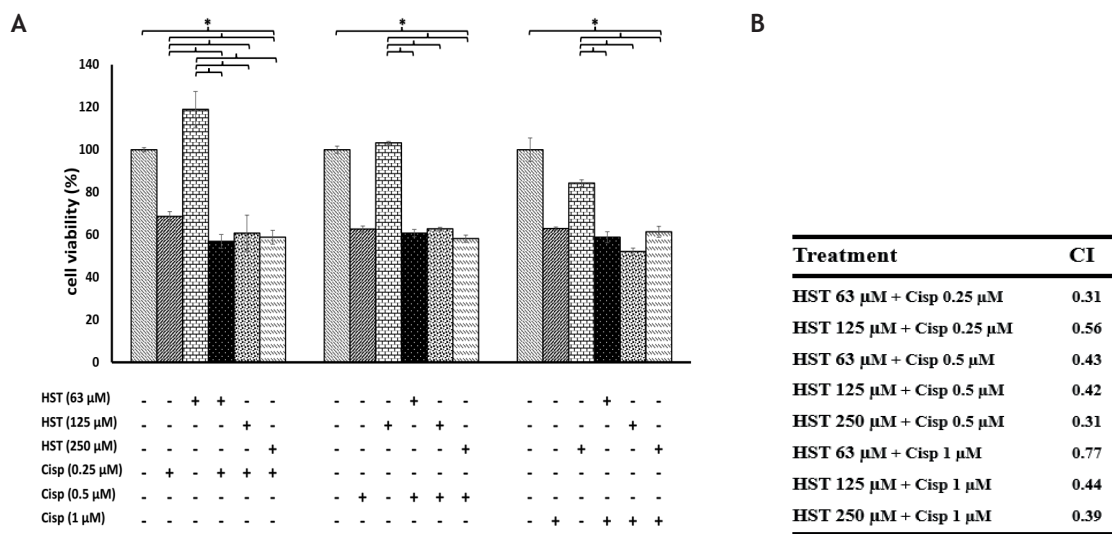


Figure 2. The effect of hesperetin (HST) and cisplatin (Cisp) to 4T1 cell's viability. (A) This effect was assessed by examining cell viability following treatment with HST in combination with Cisp. 4T1 cells (8×10^3 cells/mL) was exposed to HST concentrations 63 μM, 125 μM, 250 μM and Cisp 0.25 μM, 0.5 μM, 1 μM for a duration of 24 h and subsequently assessed using the MTT assay. (B) The combination index (CI) values for the treatment with HST and Cisp. The cell viability profile was presented as the average $\pm$ standard error (SE) from three experiments with $p < 0.05$.

Selectivity Index (SI) is obtained from the IC_{50} value of a compound against normal cells divided by the IC_{50} value of cancer cells (Aljewari, *et al.*, 2010). Compounds are classified as high selectivity if the SI value is >3 and less selective if the SI value is <3 (Sutejo, *et al.*, 2016). These results highlight the specific cytotoxic effect of HST on cancer cells found to be less selective for tested cells ($SI < 3$). Subsequently, a cytotoxicity test was carried out to assess the effect of HST on the ability of Cisp to inhibit the growth of 4T1 cancer cells. Subsequently, a combination cytotoxic test was conducted to assess the impact of HST on Cisp in inhibiting the proliferation of 4T1 cancer cells. The concentration applied on combination chemotherapy of HST was referred to IC_{50} value of each compound. There are three concentration series for each compound, *i.e.*, $\frac{1}{2} IC_{50}$, $\frac{1}{4} IC_{50}$, and $\frac{1}{8} IC_{50}$ (Meiyanto, *et al.*, 2019). The findings demonstrated that HST augmented the cytotoxic effect of Cisp on 4T1 cells (Figure 2A). The collective results indicated that HST increased

the cytotoxic potency of Cisp was statistically significant ($p < 0.05$). The combination of HST and Cisp exhibited a synergistic effect, as evidenced by a combination index (CI) value of <1 (Figure 2B). These results from the synergistic combination suggest that HST enhances the cytotoxic efficacy of Cisp against 4T1 cells.

Confirmation of Cellular Senescence: Substantiation in Vero Cells Following Hesperetin (HST) Treatment

The utilization of natural products as potential anticancer agents often impacts not only cancer cells but also normal cells. To affirm the selectivity of HST's effects on cancer cells, we examined its influence on senescence phenomena in normal kidney cells, specifically using Vero cells. The outcomes of our investigation demonstrated that HST treatment did not induce any changes in β -galactosidase-positive cells compared to untreated cells. (Figure 3A). In contrast, Cisp

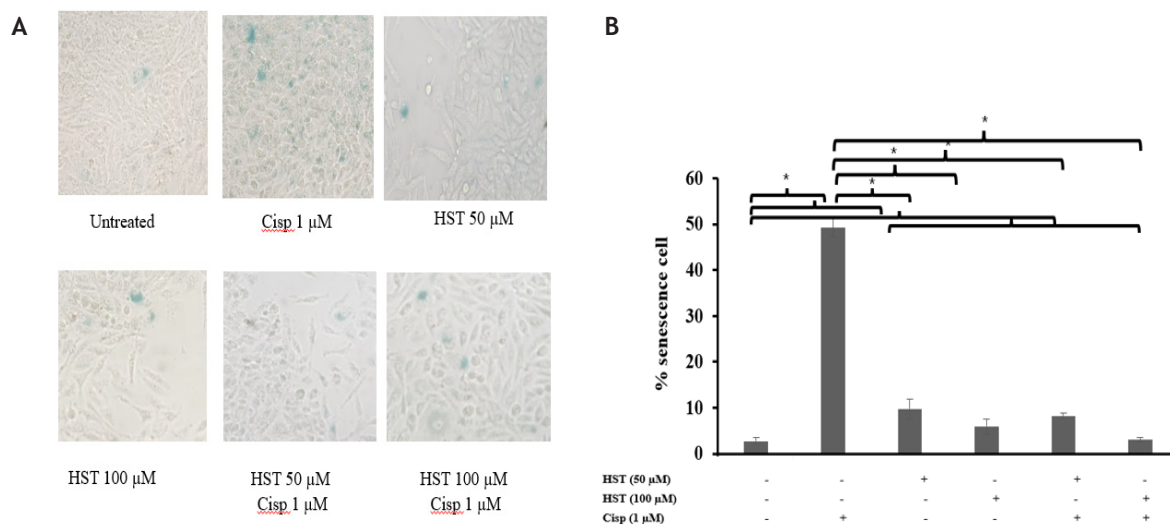


Figure 3. Cellular Senescence in Vero cells following treatment with hesperetin (HST). The analysis of senescent cells was conducted using the SA- β galactosidase staining assay. Vero cells were used as the normal cell line (2×10^4 cells/mL) and were treated with HST at 50 μ M, either single or in combination with cisplatin (Cisp) (1 μ M), for a duration of 24 h. Following this, the assessment was conducted using β -galactosidase staining. As a positive control, cells were exposed to Cisp at a concentration of 3 μ M for 24 h. The percentages of senescent cells (β -galactosidase-positive cells) were calculated ($n=3$). (A) Cellular morphology of Vero cells after 72 h of staining, observed under an inverted microscope at a magnification of 200x. (B) Quantification of senescent Vero cells.

significantly increased the presence of numerous β -galactosidase-positive cells, indicating that Cisp promotes the senescence of the Vero cells ($p < 0.01$). Furthermore, when cells were treated with Cisp and HST, a notable decrease in the number of β -galactosidase-positive cells was observed between control or Cisp and the combination, and this reduction was statistically significant ($p < 0.01$). (Figure 3B). These findings suggest that HST rescue the normal kidney cells from senescence induced by Cisp.

DISCUSSION

HST has been investigated for its diverse pharmacological advantages, particularly its potential as an anticancer agent. Nonetheless, it is crucial to comprehend its impact on normal cells. With this consideration, our main goal was to evaluate the evidence-based cytotoxic effects and physiological changes induced by HST on TNBC cells, comparing these effects with normal kidney cells. In this study, Cisp was chosen as a model chemotherapeutic agent due to its mechanism of inhibiting the growth of TNBC cells. However, it is associated with side effects such as the accelerated senescence of normal cells and resistance issues in patients (Vyas, *et al.*, 2014). These side effects are primarily acknowledged because Cisp increase intracellular ROS levels, leading to the induction of senescence in exposed cells (Pan, *et al.*, 2015). This increase may be associated with Cisp's ability to induce oxidative stress in normal cells (Yu, *et al.*, 2018). Due to its general and non-selective mechanism, the utilization of Cisp poses a risk to normal cells, particularly normal kidney cells. Therefore, this study examined the impact of HST and Cisp co-treatment on Vero cells, as a model for normal kidney cells. HST exhibited a cytotoxic effect on 4T1 cells. In a single treatment, HST exhibited moderate cytotoxicity against 4T1 cells with IC_{50} of 498 μ M. On the other hand, Cisp produced a strong cytotoxicity effect with IC_{50} of

2 μ M. The results of this study are in line with research by Yunita, *et al.*, 2020 which reported that the IC_{50} values of Hst and Dox were 400 μ M and 0.5 μ M respectively (Yunita, *et al.*, 2020). The findings indicated that HST exhibited cytotoxicity towards TNBC breast cancer cells. Interestingly, when applied to concentrations up to 1000 μ M, HST did not induce cytotoxicity in Vero cells with IC_{50} value > 500 μ M. These observations suggest a higher selectivity of HST for cancer cells than for normal cells.

HST showed a synergistic effect in combination with Cisp in 4T1 cells. These two combinations synergistically with a $CI < 1.0$. The demonstrated cytotoxic effect against 4T1 cells suggests that HST holds the potential for further development as a co-chemotherapeutic agent with Cisp to inhibit TNBC. This research is confined to the TNBC cell model derived from mice. Therefore, additional investigations are warranted, particularly using TNBC breast cancer models derived from humans, to ensure comprehensive development and applicability. However, the 4T1 cells used in this study are easily to metastasis, therefore, it is essential to observe the impact of HST in combination with Cisp not only on cell proliferation but also on migration ability. This presents an interesting challenge for future research.

This research found that HST strongly inhibits the senescence evidence in the normal kidney cell model, Vero with no cytotoxic effects. In this study, Cisp was used as an agent to induce cellular senescence in Vero cells (Li, *et al.*, 2019). The use of Vero cells could represent the pathological event by which the damage and senescence of normal kidney cells. Examination of changes in cellular physiology resulting from HST administration may reveal its preference for cancer cells. Nephroprotective is necessary nowadays because senescence problems can cause damage to vital tissues, characterized by increased activity of SA- β -Gal. However, cancer cells have the ability to avoid this process. This presents a new

alternative explore the nephroprotective potency of HST on kidney cells induced by cisplatin using the Vero cell line. Our data support shows that HST treatment increases cytotoxic Cisp in 4T1 cells but not in Vero cells. More interestingly, HST inhibits senescence in normal kidney cells in response to Cisp treatment. The Cisp used in this investigation is a chemotherapy medication recognized for its capacity to increase ROS levels and promote senescence in cancer cells (Kleih, *et al.*, 2019). HST is used in this study as a representative synthetic natural product. It has demonstrated a counteractive effect when combined with Cisp, affecting both cancer cells and normal cells. These observed effects provide support for the potential utility of HST as a co-chemotherapeutic agent with Cisp. Specifically, HST has the capacity to enhance the cytotoxic impact of Cisp on TNBC cells while concurrently mitigating its adverse effects on normal cells. As a result, further investigation into the underlying molecular mechanisms is necessary to gain a comprehensive understanding of the evidence-based therapeutic applications of HST. Further observations related to the senescence effect revealed that HST had the capacity to reduce the number of senescent cells in normal fibroblast cells, even when they were induced by stressors like Cisp (Qu, *et al.*, 2013). Therefore, the ability of HST to reduce senescence is very promising in maintaining the continuity of normal cell replication, ultimately contributing to the improvement of overall cell quality. This possibility should be further investigated by exploring additional markers associated with cytotoxicity and anti-senescence.

CONCLUSION

In summary, HST has low cytotoxicity against the Vero normal kidney cell line. HST decreased cell senescence levels in cisplatin-induced stress oxidative Vero cells. Therefore, HST has the potential as a nephroprotective agent. Nevertheless, the

underlying mechanism of these phenomena need clarification, especially concerning the relationship between cytotoxicity and anti-senescence. In conclusion, the overall findings suggest that HST has the potential to function as a co-treatment agent and may also offer an alternative option for anti-senescence in healthy tissues.

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