

The Effect of Brazilin from *Caesalpinia sappan* on Cell Cycle Modulation and Cell Senescence of T47D Cells

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ABSTRACT

Ethanol extract and brazilein-containing fraction of *Caesalpinia sappan* L., has been reported to inhibit cell proliferation on T47D (estrogen receptor positive, luminal A subtype model). The luminal A subtype is the most prevalent subtype of breast cancer in Indonesian females. In this study, we explored the activity of the reduced form of brazilein, brazilin, on T47D cells proliferation and the mechanism that involved. The cytotoxicity activity of brazilin was observed using the MTT assay. The cell cycle profile was analyzed by using flow cytometry, and the cells senescent was observed using S-A- β -galactosidase assay. The results showed that brazilin was cytotoxic to T47D with an IC₅₀ value of 50 μ M (14.3 μ g/mL). Cell cycle profile showed that after treated with brazilin, the cells were accumulated at the G2/M phase. Furthermore, cells treated with a combination of brazilin and doxorubicin were accumulated at the G2/M and sub G1 phase. Cells accumulation at sub G1 phase indicates that the cells underwent apoptosis. Our data of S-A- β -galactosidase assay showed that cells treated with $\frac{1}{4}$ IC₅₀, $\frac{1}{2}$ IC₅₀, and IC₅₀ brazilin had lower senescent cells than the untreated cells. The morphology of cells treated with IC₅₀ (50 μ M) brazilin was changed. The cells shape became rounded, cells were shrink and detached from the well plate, indicating that cells may undergo apoptosis. These results suggested that brazilin cytotoxic towards T47D cells, decreased cell senescence, and may induce apoptosis. Therefore, we believe that brazilin is potential to be further examined for its mechanism of action in inhibiting the proliferation of T47D cells.

Keywords: brazilin, *Caesalpinia sappan*, T47D, cell cycle, cell senescence

INTRODUCTION

Breast cancer is the most prevalent cancer in women around the globe, including in Indonesia. Based on the data of Globocan 2018, breast cancer positioned in the first rank of cancer incidence and mortality-causing cancer in Indonesian women (Global Cancer Observatory, 2018). The luminal A (ER⁺ PR⁺/HER2⁻) subtype is the most prevalence of breast cancer in Indonesia (Rahmawati *et al.*, 2017). Cancer cells must evade programs that negatively regulate cell proliferation to maintain their growth. Cancer cells with mutated or non-functional of tumor suppressor genes are thus missing a crucial checkpoint of cell-cycle progression, implicating in the cell over-proliferation (Hanahan and Weinberg,

2011). There is another mechanism known to halt cell proliferation besides apoptosis (programmed cell death), named cellular senescence. Cellular senescence is one of cellular homeostasis mechanism as a response to cellular stress that is defined as a permanent cell cycle arrest (Childs *et al.*, 2014; Myriantopoulos *et al.*, 2019). Senescence now becomes a target in cancer control. It was first explained in 1961, by Hayflick and Moorhead, as a replicative senescent triggered by telomere attrition (Childs *et al.*, 2014). Currently, it is known that senescence could be triggered by various extrinsic factors, including conventional chemotherapeutic agents, oxidative stress, and genetic manipulations as well. This form of

senescence was termed as stress-induced premature senescence (Myriantopoulos *et al.*, 2019), and this can be induced in tumor cells (Roninson, 2003).

It was suggested by Uchida *et al.* (2013) that the application of chemotherapy on patients with luminal A breast cancer may bring patients longer relapse-free periods. However, the cytotoxicity of chemotherapeutic agents such as cis-platinum, paclitaxel, cyclophosphamide, 5-fluorouracil, and doxorubicin (Dox) is not limited to cancer cells but also affected normal tissue (Tiwari *et al.*, 2011). The drawback leads to the development of natural compounds present in the nutrition as these phytochemicals have been displayed a chemoprevention and anticancer effects. Most of the phytochemicals are nontoxic compared to chemotherapeutic agents (Tiwari *et al.*, 2011). *C. sappan* (Sappan wood; local name in Indonesia is "Secang") is a well-known herbal plant in Southeast Asia (Nirmal *et al.*, 2015). Previously our group observed that the ethanolic extract of *C. sappan* induced apoptosis in T47D (a luminal A model breast cancer cell line) cells and had a synergistic effect with doxorubicin in inhibiting the growth of the cells (Nurzijah *et al.*, 2012). Brazilin and brazilein are two major bioactive compounds of *C. sappan*. The brazilein-containing fraction of *C. sappan* L., has been reported to inhibit cell proliferation on T47D (Tirtanirmala *et al.*, 2015). Therefore, it is important to clarify the potency of brazilin on the luminal A model breast cancer cell line. In this report, we studied the significance of brazilin from *C. sappan* in inhibiting proliferation of T47D cells as a model of Luminal A subtype breast cancer. Furthermore, as T47D carries mutated and non-functional form of *p53* (Mumcuoglu *et al.*, 2010), a critical suppressor gene that regulates cell cycle checkpoints, apoptosis, and senescence (Speirs *et al.*, 2011), we analyzed brazilin effect on cell cycle modulation and cell senescence as one of mechanisms that halt cell proliferation.

MATERIAL AND METHODS

Chemicals

Brazilin was isolated from *C. sappan*, L. using the previously reported method (Jenie *et al.*, 2018). Doxorubicin was purchased from Harlem (The Netherlands, imported by Combiphar, Indonesia).

Cell culture

The T47D cell line was a collection of Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada, which was

given by Prof. Dr. Masashi Kawaichi (Nara Institute of Science and Technology, Japan). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA) with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), and 1% penicillin-streptomycin (Thermo Fisher Scientific).

Cytotoxicity assay

The T47D cells (1×10^4 /well) in 96-well plates were treated with brazilin at 5-100 μ M. The sample was prepared at concentration 5mg/mL (17 μ M) with a dimethyl sulfoxide (DMSO) as co-solvent, and then diluted in DMEM cell culture medium. Cells were incubated for 24h at 37°C and 5% CO₂, then cells were washed in phosphate-buffered saline (PBS) (Sigma-Aldrich). Cells were incubated with 5mg/mL MTT reagent (Sigma-Aldrich) for 4h then the reaction was stopped using 10% sodium dodecyl sulfate (SDS) in 0.01M HCl (Millipore Sigma) overnight. The absorbance was measured at 595nm. The results were presented as a percentage of cell viability. The concentration of sample that inhibited 50% of cell viability of the control DMEM (IC₅₀) was calculated from three independent experiments in triplicate.

Cell cycle analysis

The T47D cells (5×10^4 cells/well) were grown in the 24-well plate, treated with various concentrations of samples (brazilin and doxorubicin) for 24h. Cells were then harvested, fixed with 70% ethanol and stained with propidium iodide (PI)/RNase staining kit (BD Biosciences, San Jose, CA) to analyze the DNA content using flow cytometry (BD Biosciences Accuri C6) and BD Accuri C6 software.

Senescence-associated β -galactosidase assay

Senescence-associated β -galactosidase (SA- β -gal assay) was used to observe the senescent cell. The method was performed as previously described (Hanif *et al.*, 2019). Briefly, T47D cells were grown at 1×10^4 cells in 6-well plate and treated with samples (brazilin and doxorubicin) for 24h. Cells were washed and fixed then incubated in staining solution for 48h at 37°C CO₂ free. Cells were observed using an inverted microscope (Olympus CKX41) at 100x magnification. The β -D-galactosidase positive cells were quantified using ImageJ software. The results were presented as a percentage of senescent cell, which was calculated from positive cells compared to the total cell number in one observing field.

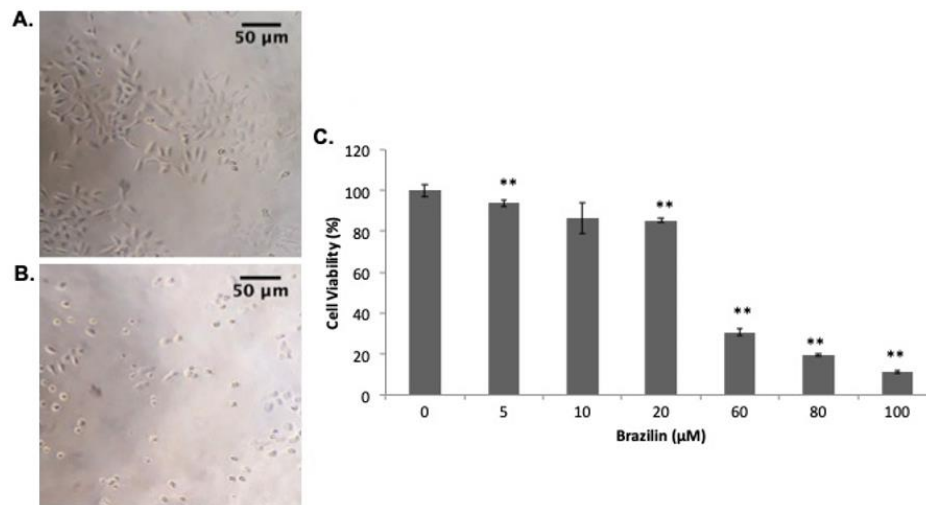


Figure 1. Cytotoxic activity of brazilin on T47D cells. Cells morphology after treatment of untreated (A) and 60µM brazilin (B). Percentage of T47D cell viability after samples treatment (C). Cells were treated with 5-100µM of brazilin for 24h before assessed by MTT assay. Error bar represents the standard deviation from three independent experiments. ** $P < 0.01$.

We performed calculation from three different observing fields from each experiment.

Data analysis

All data were expressed as means \pm SD or SE. Statistical analysis of the experimental data was conducted by using Student's t-test (Excel 2013 software; Microsoft, Redmond, WA). P values of less than 0.05 or 0.01 were considered to be significant.

RESULTS AND DISCUSSION

Cytotoxicity of brazilin on T47D cells

Our group has reported the cytotoxicity of ethanolic extract of *C. sappan* on T47D with the IC_{50} value of 35µg/mL (Nurzijah *et al.*, 2012). T47D cells viability was decreased after treatment of brazilin for 24h in a dose-dependent manner (Figure 1). Cell morphology was changed under treatment of brazilin. The increased brazilin concentration caused shrinkage of the cells, cells round shape, and detached from the culture plate (Figure 1 A-D). These morphology changes were suggested to be the characteristics of cells that undergo apoptosis (Saraste and Pulkki, 2000).

Cell cycle modulation of brazilin on T47D cells

The cell proliferation relies on the cell cycle progression. Thus, we investigated the outcome of brazilin on cell cycle modulation (Figure 2). We used doxorubicin (Dox) as a positive control

because Dox is a chemotherapeutic agent that is known to induce G2/M arrest in T47D cells (Meiyanto *et al.*, 2011). The IC_{50} of Dox on T47D was 50nM, according to Jenie and Meiyanto (2007). We confirmed that Dox (25 and 50nM) halt the T47D cell cycle progression at G2/M phase (Figure 2D,E). Meanwhile, we observed that brazilin caused cell accumulation at G2/M phase and increased sub G1 population in a dose-dependent manner (Figure 2B-2C). Cells that were given with the combination of $1/2IC_{50}$ brazilin (25µM) and $1/2IC_{50}$ doxorubicin (25nM), were accumulated at G2/M phase and sub G1 phase, increased around 25% and 10%, respectively, compared to untreated cells (Figure 2E).

Brazilin effect on cellular senescence of T47D

T47D is a well-known breast cancer cell with mutated and non-functional *p53* (Mumcuoglu *et al.*, 2010). The *p53* protein is a critical controller of cell cycle checkpoints, apoptosis, and senescence (Speirs *et al.*, 2011). Therefore, we conducted an S-A- β -galactosidase assay to assess the effect of brazilin on T47D cellular senescence. The results showed that untreated T47D contained 30% senescent cells as quantified in the graph (Figure 3A, G). The senescent cells in the untreated cells may be related to the fact that tumor cell senescence could develop spontaneously in response to the environment changes.

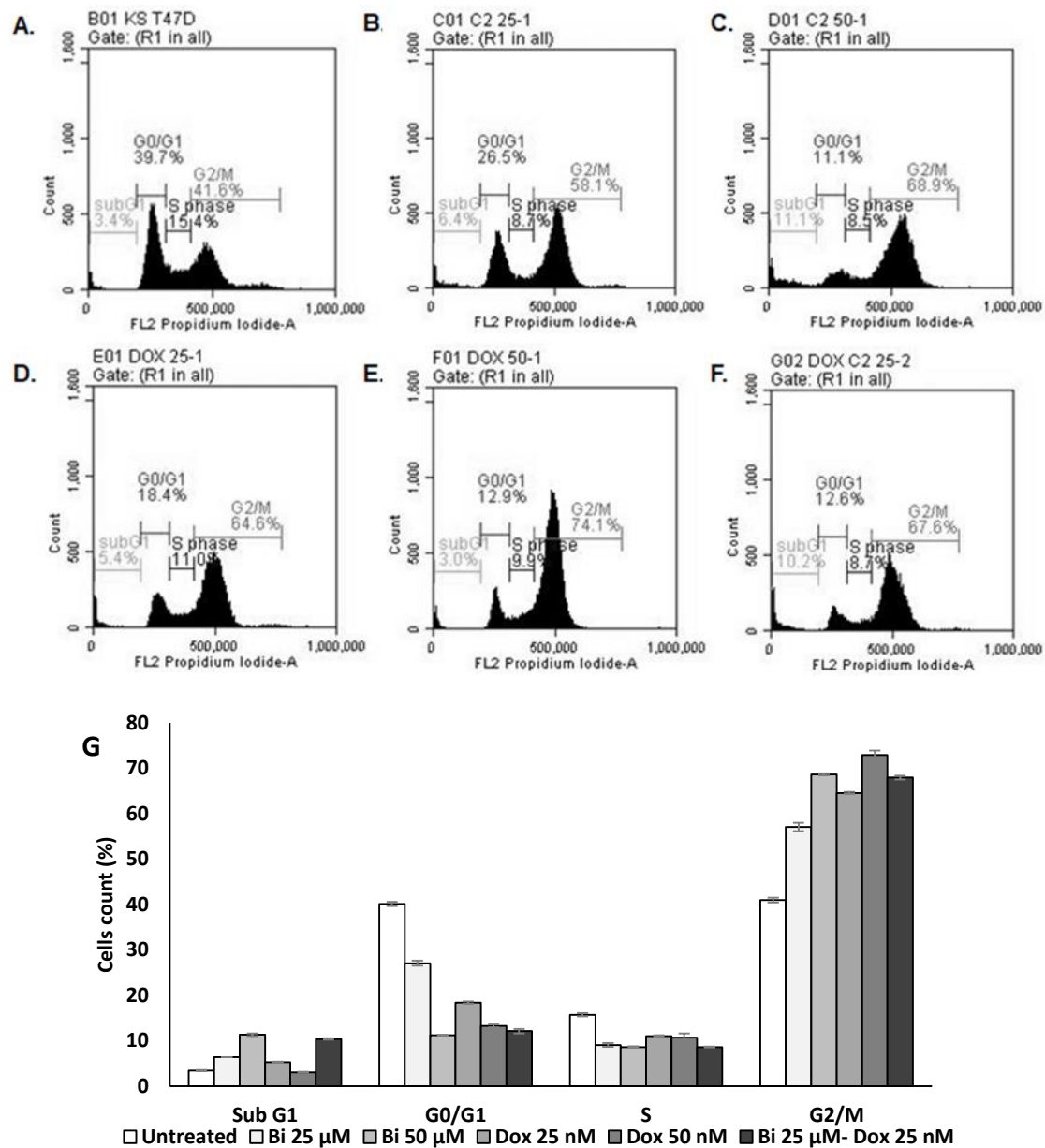


Figure 2. The effect of brazilin on T47D cell cycle profiles. Histogram profile of T47D cells after treated with untreated (A), 25μM brazilin (Bi) (B), 50μM Bi (C), 25nM doxorubicin (Dox) (D), 50nM Dox (E), or combination of 25μM Bi and 25nM Dox (F) for 24h, then stained with PI/RNase for cell cycle analysis. (G) Cell cycle profile of T47D cells. The analysis of the cell cycle was conducted using flow cytometry as described in the Materials and Methods. Error bar represents the standard error from three experiments.

The senescent cell was observed in approximately 20% of the tumor cells, even without drug treatment (Roninson, 2003). Moreover, according to a study by Mumcuoglu *et al.* (2010), T47D cells were categorized into senescent cell progeny (SCP) due to their capability to generate senescent progeny in a low-density clonogenic circumstances.

As cell senescence could be induced by treatment of chemotherapeutic agents (Roninson, 2003), we treated the cells with Dox to compare it with brazilin. Our study showed that 10nM Dox increased 20% of senescent cells compared to untreated cells (Figure 3B,G). In contrast, 50nM Dox decreased senescent cells until less than 10%

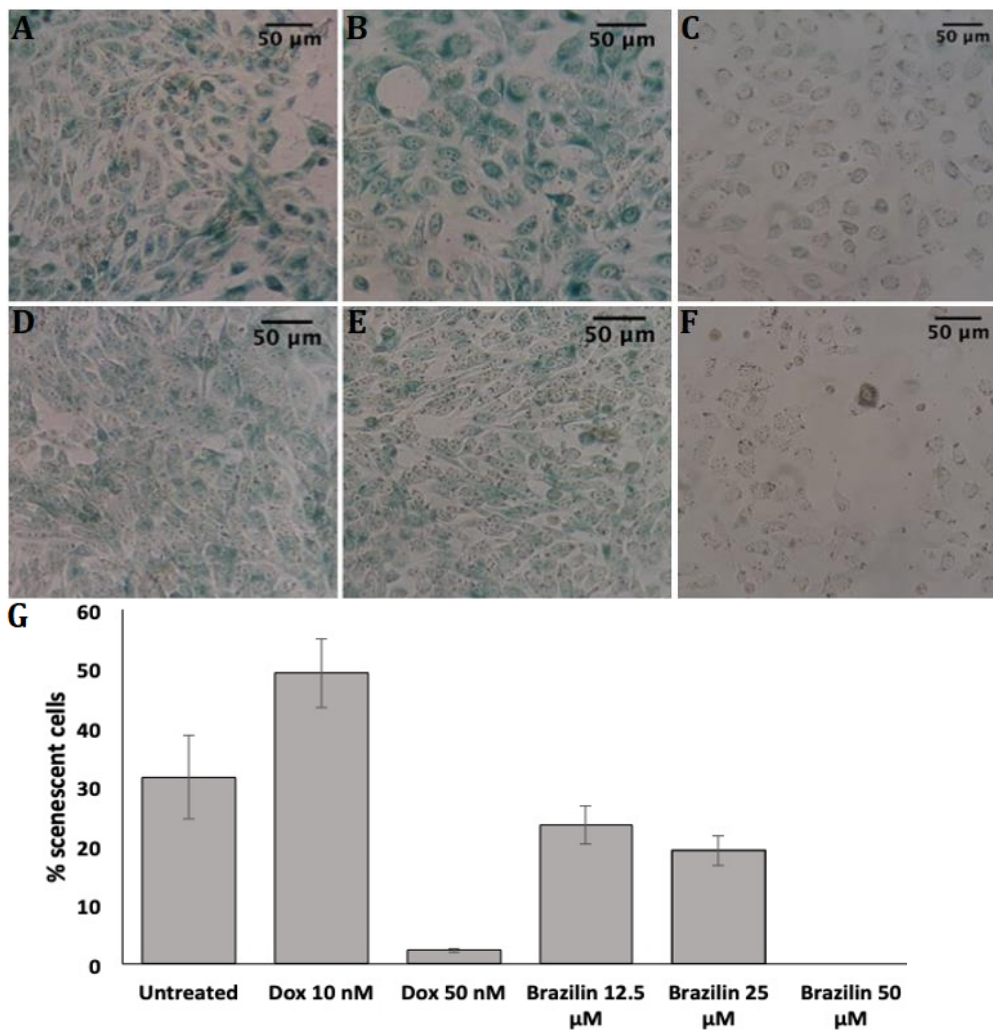


Figure 3. The effect of brazilin on T47D cellular senescence. T47D cells were grown at 1×10^4 cells, except the untreated (A), the cells were then treated for 24h with 10nM doxorubicin (Dox) (B), 50nM Dox (C), 12.5μM brazilin (Bi) (D), 25μM Bi (E), and 50μM Bi (F). Cells were then stained by the substrate solution of the senescence-associated β -galactosidase for 48 h resulting in blue-green color cells. Senescence evidence was monitored by an inverted microscope in 100x magnification and quantified as described in the Materials and Methods (G). Error bar represents the standard error of quantification from three observing fields.

(Figure 3C,G). These data were indicating that the concentration of the chemotherapeutic agent determined cell fate. At low concentration, Dox was reported to induce intracellular the reactive oxygen species (ROS) level and senescence (Hanif *et al.*, 2019; Meiyanto *et al.*, 2018). Low concentration of Dox was reported to promote senescence in SKN-SH neuroblastoma and colorectal carcinoma cells, associated with high *p21* expression, whereas high concentration of Dox resulted in low *p21* expression and apoptosis (Childs *et al.*, 2014). Moreover, brazilin (at $\frac{1}{4}$ IC_{50} ,

$\frac{1}{2}$ IC_{50} , and IC_{50}) reduced cell senescence in a dose dependent manner (Figure 3D-3G). Brazilin at $\frac{1}{4}$ IC_{50} decreased cell senescence by 10% compared to the untreated and at IC_{50} , the senescent cells were very low, similar to that upon 50nM Dox.

In the current study, we evaluated the anti proliferation of brazilin against T47D cell line. The MTT assay demonstrated that brazilin was cytotoxic with an IC_{50} value of 14.3μg/mL (50μM) (Figure 1E). The value was lower than the IC_{50} of the ethanolic extract of *C. sappan* and the brazilein-containing fraction on the same cell line which

were 35µg/mL and 68µg/mL, respectively (Nurzijah *et al.*, 2012; Tirtanirmala *et al.*, 2015). Brazilin and brazilein are the major components of *C. sappan* with a similar structure. Moreover, brazilein is the oxidized form of brazilin (Rondão *et al.*, 2013). The lower IC₅₀ value of brazilin compared to brazilein, indicating that brazilin was more potent than brazilein against T47D cells. The capability of brazilin to reduce the growth of other cancer cell type was observed by our group. The IC₅₀ of brazilin in WiDr and MCF-7/HER-2 were 41 µM and 54µM, respectively (Handayani *et al.*, 2017; Jenie *et al.*, 2018). However, brazilin was also reported to be cytotoxic against normal fibroblast cells (NIH 3T3 and MEF) (Lee *et al.*, 2015), suggesting a consideration on brazilin selectivity.

Based on the cell cycle analysis we observed that brazilin caused cell accumulation on subG1 and G2/M phase (Figure 2B,C). The sub G1 phase population is often related to apoptotic cells, as one of the apoptotic characteristics is DNA fragmentation, which results in a reduced content of DNA (Studencka and Schaber, 2017). This result was suggesting that the cells were directed into apoptosis via G2/M arrest. The ethanolic extract of *C. sappan* has already reported inducing apoptosis up to 60% in T47D cells compared to untreated cells by Nurzijah *et al.* (2012). Other researchers also reported that brazilin also induced G2/M arrest on several other cancer cell types including U266 cells, WiDr, and MCF-7/HER2 (Kim *et al.*, 2012; Handayani *et al.*, 2017; Jenie *et al.*, 2018). Kim *et al.* (2012) suggested that the mechanism underlies G2/M arrest by brazilin involving increased the level of cyclin-dependent kinase (CDK) inhibitor, particularly the p21 and p27 in a time-dependent manner. As a result, the level of cyclin B1 and E were reduced after 24 or 48h of brazilin treatment and stimulated the regulator proteins of G2 checkpoint, Chk1 and Chk2, in U266 cells.

In the present study, we observed that brazilin reduced cell senescence in a dose-dependent manner (Figure 3D-3G). Previous work from our group reported that treatment of ethanolic extract of *C. sappan* on 4T1 cells gave non-significant cell senescence compared to untreated cells (Hanif *et al.*, 2019). Furthermore, according to that study, ROS level of 4T1 cells treated with brazilin was decreased. The addition of ethanolic extract of *C. sappan* on Dox or hydrogen peroxide treated cells was reported to significantly decrease the intracellular ROS level compared to the single treatments of Dox or hydrogen peroxide on CHO-

K1 cells (Meiyanto *et al.*, 2018). ROS is known as one of the inducers of cell senescence (Myriantopoulos *et al.*, 2019). High level of ROS plays a vital role in inducing and maintaining cell senescence (Davalli *et al.*, 2016).

Many reports suggested the antioxidant activity of *C. sappan* and its constituents, as reviewed by Nirmal *et al.* (2015). Amongst other compounds from Sappan lignum, brazilin exhibited the utmost DPPH radical scavenging activity and ferric reduction activity and it is even higher compared to standard vitamin E (Sasaki *et al.*, 2007). Furthermore, *C. sappan* extract and brazilin were also known to scavenge UVA-induced secretions of hydrogen peroxide and increase the expression of the glutathione peroxidase 7 (GPX7) enzyme (Hwang and Sim, 2018). Thus, we suggest that the decreased cell senescence under brazilin treatment was being attributed to its activity as an antioxidant which causing the level of ROS was not high enough to induce cell senescent. However, this hypothesis needs to be further clarified by measuring the ROS level and observing the expression level of antioxidant enzymes in T47D cells under treatment of brazilin.

CONCLUSION

Our current study revealed that in T47D cells, as a model of Luminal A subtype breast cancer, brazilin was cytotoxic and induced cell cycle arrest at G2/M phase and possibly directed cells to undergo apoptosis as indicated by the increasing cell population at sub G1 phase. We also demonstrated that brazilin decreased cell senescence in a dose-dependent manner.

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