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Alpinia galanga extract induces senescence in human epidermal growth factor receptor 2-overexpressing breast cancer cells

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ABSTRACT

Background: *Alpinia galanga* L. (galangal) is a widely known spice in Southeast Asia. A previous study indicated that its active compounds might effectively inhibit (human epidermal growth factor receptor 2 [HER2])-overexpressing breast cancer cell proliferation. **Objective:** Our study aimed to investigate the antiproliferation activity of galangal extract (GE) on HER2-overexpressing breast cancer cells by analyzing GE cytotoxicity and its mechanism of action. **Materials and Methods:** We used HCC1954 cell line as a model. We observed the effect of GE (10–200 μg/mL) on cell growth using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The modulation of GE on cell cycle, apoptosis, and the intracellular reactive oxygen species (ROS) level was analyzed using flow cytometry. Moreover, SA-β-galactosidase staining used to observe the cell senescence. **Results:** Cytotoxic test indicated that GE has modest cytotoxicity toward HCC1954 (IC_{50} >200 μg/mL) and GE increased cell accumulation at the G2/M phase. Although GE showed no induction on apoptosis, it increased the number of senescent cells. This effect correlated with the increasing intracellular ROS level on treatment with GE. Furthermore, when combined with doxorubicin (Dox), GE increased the Dox's cytotoxicity. **Conclusion:** *A. galanga* antiproliferation activity against HER2-overexpressing breast cancer was associated with its capability to induce cell senescence and intracellular ROS level, causing halted cell cycle progression.

Keywords: *Alpinia galanga*, breast cancer, human epidermal growth factor receptor 2, reactive oxygen species, senescence

INTRODUCTION

Cancer refers to the overproliferation of cells in the body; cancer cells can metastasize, leading to the mortality of 3500 million people per year around the world.[1] Breast cancer has the second-highest prevalence among cancers and is the most common and deadliest for women worldwide.[2,3] Breast cancer is classified into several subtypes; one of them is human epidermal growth factor receptor-2 (HER2) overexpression subtype which involves an extra copy of the *HER2* gene and plays a role in malignancy, causing tumor cells to become aggressive and invasive and easily spread or metastasize to lymph vascular tissues.[4] Excessive proliferative signaling, which can trigger cell senescence, is one of the hallmarks of cancer. Cellular senescence is one of the cellular homeostasis mechanisms of the response to cellular stress and is described as an irreversible cell cycle arrest.[5,6] Senescence is now a target in cancer control because it halts cell cycle and cell proliferation when the cells undergo senescence.

Approximately 20–30% of primary breast cancer overexpresses HER2.[7] At present, trastuzumab and lapatinib are used to treat the HER2 subtype of breast cancer. However, each of these drugs has drawbacks. For example, only one-third of patients respond to trastuzumab treatment, with most responders demonstrating disease development after 1 year of therapy. This report indicates that most of the patients

treated with trastuzumab develop resistance.[8] Lapatinib was reported to have specific toxicity associated with diarrhea and rash.[9] Thus, a supportive agent that is safe and has minimal side effects on the patient's body is needed to minimize the side effects of cancer drugs. Plants and herbs have various bioactive compounds that render them worthy of exploration as drug candidates. Given that plant-derived bioactive compounds have many biological properties, such properties must be identified and characterized to evaluate the potency of plants to be developed as anticancer agents.[2]

Alpinia galanga L. belongs to the Zingiberaceae family and is used as a traditional spice in Southeast Asian countries. Many researchers widely studied the active compounds from the various parts of *A. galanga*. 1'-Acetoxychavicol acetate (ACA) is one of the compounds present in *A. galanga* rhizomes; it inhibits the tumor formation induced by chemical compounds and suppresses the growth of Ehrlich ascites tumor cells.[10] Other groups also observed that ACA suppresses Epstein–Barr virus activation *in vitro*.[11] A flavonoid presents in the root of *A. galanga*, that is, galangin (3,5,7-trihydroxyflavone), inhibits the growth and the metastasis of B16F10 melanoma cells.[12] Another compound extracted from *A. galanga*, 4'-hydroxycinnamaldehide (4'-HCA), induces the production of reactive oxygen species (ROS) in HL-60 and U937 cells.[13] An increase in oxidant levels or ROS is essential in forming the senescence phenotype of cancer cells.[14] The crude extract of *A. galanga* inhibits the growth of triple-negative breast cancer (TNBC) cells by increasing senescence and the intracellular ROS level.[15]

A. galanga belongs to the Zingiberaceae family, and it has been used as a traditional medicine in the Southeast Asian region. Scientific experimental reports indicate galangal activity toward cancer cells, with the mechanisms related to cancer metabolism.[10-15] However, no evidence supports its activity toward HER2-overexpressing breast cancer cells. Therefore, this study intended to explore galangal extract (GE) activity in the proliferation of HER2-overexpressing breast cancer cells and the cellular mechanisms of action, including apoptosis, cellular senescence, and intracellular ROS level using a cell line model. This study would provide information on *A. galanga* as a supportive agent in treating breast cancer.

MATERIALS AND METHODS

Plant Materials

We acquired *A. galanga* L. rhizome from the Medicinal Plant and Traditional Medicine Research and Development Center, The Ministry of Health, Republic of Indonesia. The plants were grown in Tawangmangu, Central Java. Five hundred grams of galangal dried simplicia were made into fine powder by grinding, then sieved using a 50-mesh sieve. We macerated with 96% ethanol as a solvent with a ratio of 1:10 for 3×24 h. During the first 6 h, the mixture was stirred occasionally. The filtrate was then concentrated with a rotary evaporator until obtained a thick extract. The GE was diluted in DMSO and made in a series of concentrations in the range of 10–200 μg/mL.

Cell Preparation

The breast cancer cell line HCC1954 (ATCC® CRL-2338) was obtained from Dr. med. dr. Muhammad Hasan Bashari, M. Kes, Laboratory of Culture and Cytogenetic Cells, Faculty of Medicine, Padjadjaran University (UNPAD, Indonesia). The cells were grown in a Roswell Park Memorial Institute 1640 (Gibco) culture medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 0.5% fungizone (Gibco, New York, USA).

Cytotoxic Assay

The cytotoxicity effect of was evaluated by performing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were grown in a 96-well plate at 9×10^3 cells/well. The cells were treated with GE, or in the combination assay, with GE and doxorubicin (Dox) (Sigma) at various concentrations, as indicated in the graph and then incubated at 37°C and 5% CO_2 for 24 h. After incubation, we discharged the media and washed the cells with phosphate-buffered saline (PBS). The cells were incubated with 5 mg/mL MTT reagent in culture medium at 37°C and 5% CO_2 for 4 h. About 10% sodium dodecyl sulfate in 0.01 M HCl (Sigma) was added to stop the reaction, and the cells were incubated at room temperature overnight. The absorbance was then determined at a wavelength of 595 nm using a microplate reader (Bio-Rad).

Cell Cycle Analysis

HCC1954 cells (2×10^5 cells/well) seeded into a 6-well plate. After 24 h, cells were treated with 50 and 100 μg/mL GE and 100 nM Dox then incubated for 24 h. Cells were then harvested and centrifuged at 2000 rpm for 3 min, continued by washing with PBS. Next, the cell pellets were stained using 50 μg/mL propidium iodide (PI)/RNase and Triton-X, then incubated for 10 min at 37°C in the dark. We analyzed the cell cycle using BD Accuri C-6 flow cytometer. The cell distribution in each phase was determined using BD Accuri C6 software.

Apoptosis Assay

Flow cytometry with Annexin V staining used to observe apoptosis. The HCC1954 cells (2.5×10^5 cells/mL) were seeded for 24 h and then treated with GE or Dox. The samples then incubated at 37°C and 5% CO_2 for 24 h. Next, the medium removed, and the cells were collected using trypsin. Detection of apoptosis cells was performed by staining the cells with Annexin V kit (Roche), which consisted of Annexin V and PI/RNase, and incubated for 10 min in a dark room. The cells were analyzed using BD C6 Accuri flow cytometer (BD Bioscience).

Senescence-associated β-galactosidase (SA-β-Gal) Assay

HCC1954 cells with a concentration of 1.5×10^5 cells/well were grown in a 6-well plate and then incubated with GE samples for 24 h. The media removed, and the cells washed with PBS. A fixation buffer was added, and the cells were allowed to stand for a certain time and then washed again with PBS. The X-Gal solution (containing reagents including 0.2% 5-bromo-4-chloro-3-inolyl-β-D-galactoside, 40 mM citric acid/phosphate buffer (pH 6.0), 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, and 2 mM $MgCl_2$) was added, and incubation was continued at 37°C for 72 h. The cells were then observed under

a microscope (CKX-41 Olympus) at 200X and captured with a digital camera. The appearance of blue cells indicated positive galactosidase cells (senescent cells).[16]

2',7'-Dichlorofluorescein Diacetate (DCFDA) Staining ROS-based Assay

HCC1954 cells (5×10^4 cells/well) seeded in 24-well plates for 24 h. After 24 h, cells were collected and placed in a sterile microtube with the supplemented buffer. The cells trypsinized with trypsin-0.25% EDTA (Gibco). The reaction inactivated by adding 500 µL ×1 supplemented buffer. Cells were then stained with 10 mM DCFDA (Sigma) and then incubated at 37°C and 5% CO_2 for 30 min. The cells treated with GE in a single treatment and combination with Dox for 4 h. Analysis of intracellular ROS performed using BD Accuri C6 flow cytometer (BD Bioscience) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Statistical Analysis

Statistical analysis was done using SPSS 20 software. The results from each assay were expressed as mean±SD or mean±SE. One-way analysis of variance (ANOVA) and *post-hoc* least significant difference (LSD) test were used to determine the statistical significance. Statistical significance was considered at $P < 0.05$.

RESULTS

Cytotoxicity and Cell Cycle Analysis on GE Treatment

To determine the effect of GE on HER2-overexpressing breast cancer cell proliferation, we performed cytotoxic analysis using MTT assay. We used HCC1954 cells, a wild-type HER2-overexpressing cell line. The cytotoxic effect was evaluated based on the percentage of cell viability after GE treatment for 24 h compared with untreated cells. We observed that GE, with a 10–200 µg/mL concentration, reduced cell growth in a dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) of GE was more than 200 µg/mL [Figure 1a]. This indicated that GE exhibits modest cytotoxicity against HER2-overexpressing cells as IC_{50} values of an extract with potent cytotoxicity are in the range of 10–100 µg/mL.[17] To explore the mechanism of GE in inhibiting HCC1954 cell proliferation, we performed cell cycle analyses. We treated the cells with 50 and 100 µg/mL GE and analyzed the cell cycle modulation. We chose 50 and 100 µg/mL because cell viability was already significantly decreased compared to untreated cells at these concentrations. However, the percentage of viable cells was still high (>80%) indicated that both concentrations were sub IC_{50}. The results showed that GE treatment (50 and 100 µg/mL) halted cell cycle progression at the G2/M phase [Figure 1b and c]. These data suggested that although cell viability was still high on treatment of 50 and 100 µg/mL GE, cell proliferation was halted, and cells arrested at the G2/M phase. Therefore, we need to explore whether this condition led the cells to apoptosis by conducting apoptosis assay.

Effect of GE on Cell Apoptosis

Next, we tested the effect of GE on apoptosis to determine further the mechanism that underlies the decreased cell viability upon treatment of GE. Based on the characteristic features, cell death occurred as apoptosis and necrosis. Figure 2 shows that on treatment with 50 and 100 µg/mL

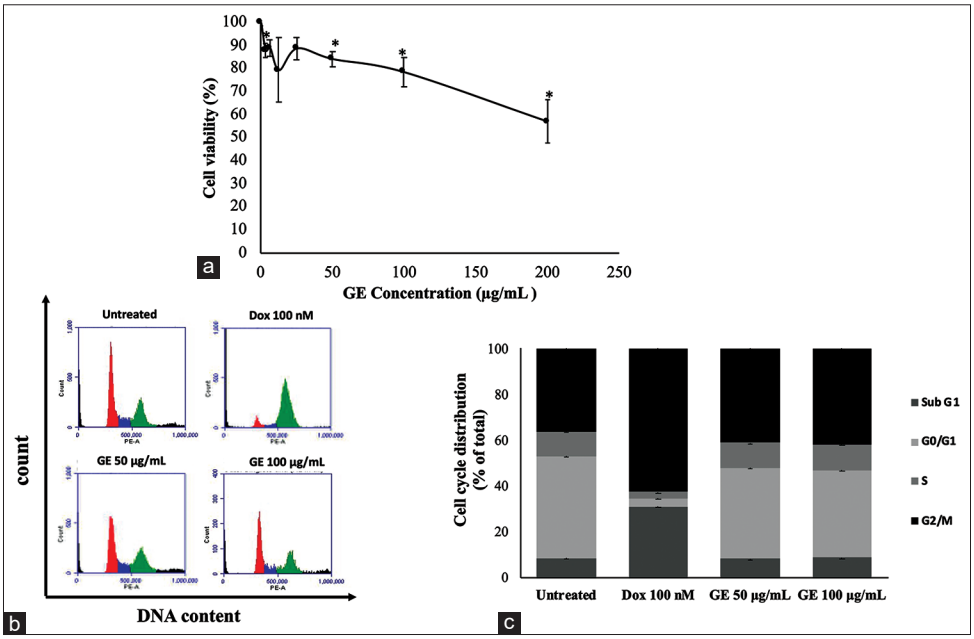

Figure 1: Cytotoxicity of galangal extract (GE) and cell cycle analysis on GE treatment toward HER2-overexpressing cells. (a) HCC1954 cells incubated with GE (10–200 μg/mL) for 24 h. The cytotoxicity of GE was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (b) HCC1954 cells treated with 50 and 100 μg/mL GE or 100 nM Dox for 24 h, stained with propidium iodide/RNase, and proceeded to flow cytometer. Cell cycle analysis conducted using flow cytometry, as described in Materials and Methods. (c) Graph of the cell cycle analysis. Error bar represents the standard deviation from three independent experiments. *$P < 0.05$ by least significant difference test

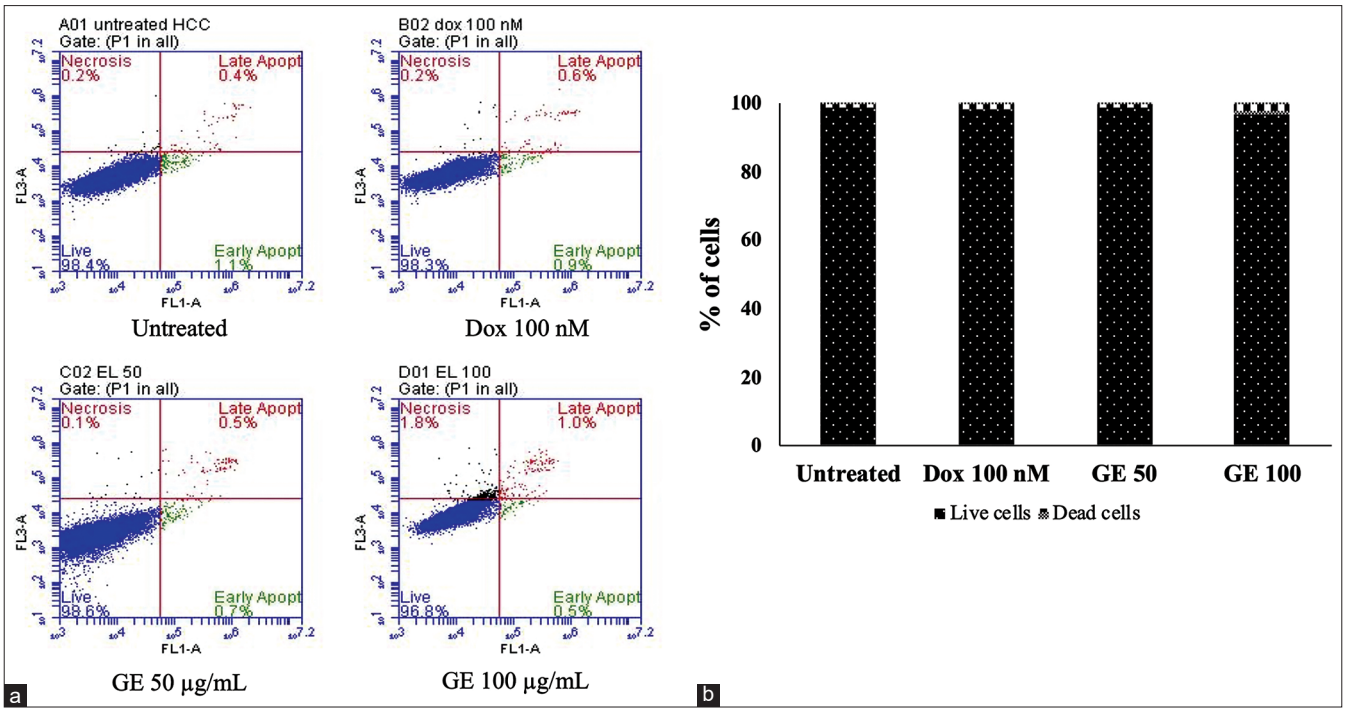

Figure 2: Effect of galangal extract (GE) on cell apoptosis. HCC1954 cells (2.5×10^5 cells/mL) were seeded for 24 h and then treated with GE, Dox, or their combination for 24 h. The cells were then stained with Annexin V/propidium iodide for apoptosis analysis, as described in Materials and Methods. The cells were analyzed using BD C6 Accuri flow cytometer. (a) The chromatogram of the apoptosis profile. (b) The graph of cell apoptosis analysis

GE or 100 nM Dox, >90% of cells were detected as live cells. These findings suggested that Dox and GE at the concentration used and an incubation time of 24 h had little effect on the apoptosis of HCC1954.

Induction of Cellular Senescence Causing by GE Treatment

The apoptosis assay data led us to analyze the possibility of GE effect on cellular senescence given that senescence is another mechanism that halts cell proliferation. Senescent cells exhibit specific characteristics, including a flat, larger form, and elevated SA-β-Gal activity. The cell cycle of such cells is in the irreversible arrested state, and the p53-p21 and p16-Rb signaling pathways are activated.[18] We performed SA-β-Gal assay to explore the anti-senescence activity of GE toward HER2-overexpressing breast cancer cells. We used Dox, which is known as a senescence-inducing agent, as a positive control.[19] Figure 3a shows the cell morphology under Dox and GE treatment. The treated cells, either by Dox or GE, became larger compared with the untreated ones. Dox treatment caused significant cell senescence (~6x), as shown in Figure 3b.

Moreover, GE treatments (50 and 100 µg/mL) also significantly induced cell senescence ($P < 0.05$) compared with the control cells. The increase in senescent cells under GE treatments was comparable to that under Dox treatment, indicating that galangal is a potent inducer of cell senescence. However, the combination treatment of Dox and GE increased the number of senescent cells at the same level as GE only treatment. Dox treatment increased cell population at subG1 [Figure 1c] indicated that the cells undergo apoptosis. When Dox combined with GE, some cells may become accelerated to undergo apoptosis. Therefore, the number of senescent cells seemed to be the same as in the single GE treatment. Cellular senescence is a complex process that is commonly triggered by oxidative stress. The increase in the intracellular ROS level is believed to be the leading cause of cell senescence.[20] Thus, we continued the study by investigating the changes in the intracellular ROS level under GE treatments.

Effect of GE on the ROS Level

ROS accumulation that exceeds the threshold level can cause DNA damage that proceeds to the aging phase (senescence).[20] Our results of DCFDA staining ROS-based assay of HCC1954 cells showed that GE increased intracellular ROS levels significantly compared with untreated cells [Figure 4a and b]. Dox treatment as a positive control resulted in a doubled ROS level compared with the untreated cells. Similar to GE effect on cell senescence of HCC1954, the treatment with GE at concentrations of 50 and 100 µg/mL increased the ROS level at a comparable level to that under 100 nM Dox. Furthermore, the combination treatments of Dox and GE also increased the ROS level, although not as significantly as the single treatment. This finding might implicate to the viability of the cells. Therefore, we examined the cytotoxicity of the combined Dox and GE treatment toward HCC1954. Based on the result shown in Figure 4c, the combination of 100 nM Dox and 50 and 100 µg/mL GE decreased the cell viability by up to 10% and 20%, respectively, compared with Dox treatment alone. However, a single treatment with GE caused no significant

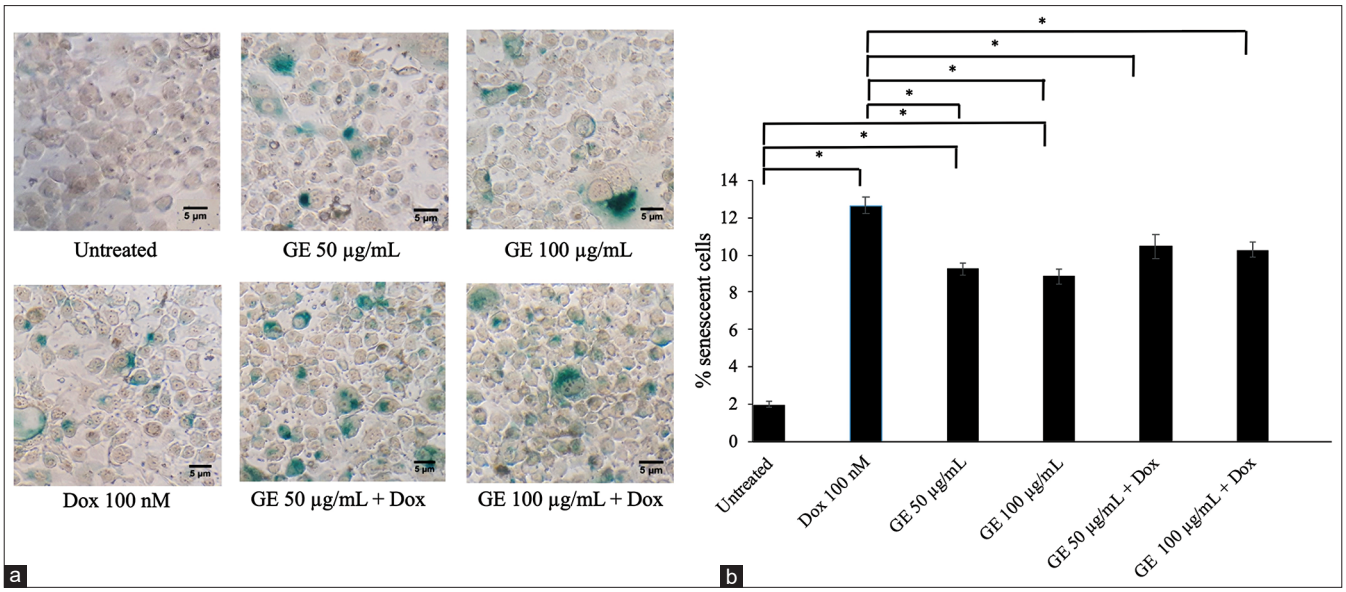

Figure 3: Induction of cellular senescence upon galangal extract (GE) treatment. HCC1954 cells at 1.5 × 10^5 cells/well were seeded and then incubated with samples or doxorubicin (Dox) for 24 h. The cells were washed and subjected to SA-β-Gal assay. The cells were stained and incubated for 72 h and then observed under an inverted microscope (×200). (a) The appearance of blue cells indicated the positive galactosidase cells (senescent cells). (b) The percentages of senescent cells (β-galactosidase-positive cells). Error bar represents the standard error from triplicate experiments, *$P < 0.05$ by least significant difference test

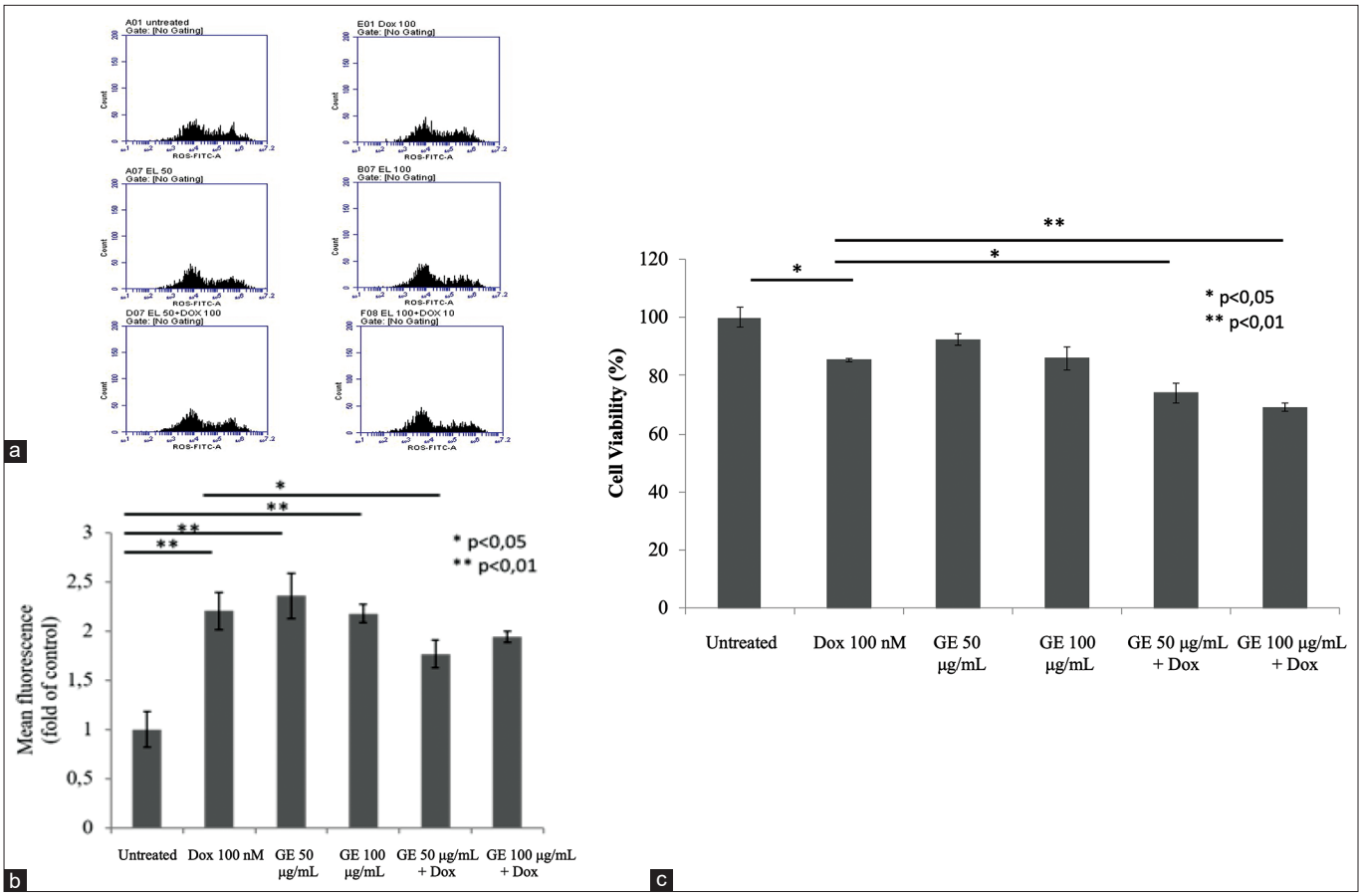

Figure 4: Galangal extract (GE) increased the intracellular reactive oxygen species (ROS) level and increased the cytotoxicity of doxorubicin. (a) HCC1954 cells were grown and stained with DCFDA fluorescence dye for 30 min and treated as the samples for 4 h, as depicted in the graph. Intracellular ROS level was measured BD Accuri C6 flow cytometer. (b) Intracellular ROS level was measured BD Accuri C6 flow cytometer. (c) Cytotoxicity assay of the combination of GE and doxorubicin (100 nM) performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described in Materials and Methods. Error bars represent standard error from triplicate experiments, *$P < 0.05$; **$P < 0.01$ by least significant difference test

decrease in cell viability. This result indicated that although GE has modest cytotoxicity toward HER2-overexpressing breast cancer cells, it showed a beneficial effect on Dox in treating breast cancer cells.

DISCUSSION

A. galanga L. has been studied worldwide for its bioactivities, including as an anticancer agent. This study aimed to evaluate the cytotoxic properties of the physiological changes caused by galangal on HER2-overexpressing breast cancer cells using wild-type HER2-overexpressing cell lines, HCC1954. First, we extracted galangal through the maceration method using 96% ethanol, an organic (non-polar) solvent capable of attracting non-polar galangal compounds, such as galangin and ACA.[21] Using thin-layer chromatography, we confirmed that the GE we used contained ACA (data not shown). Our group previously reported that GE contains compounds, such as eugenol and its derivatives, sesquiterpenes, coumarin, and other essential oils, including beta-sesquiphellandrene, beta-bisabolene, and beta-caryophyllene, which are widely reported to have cytotoxic and antioxidant properties.[15]

The IC_{50} of GE in HCC1954 cells was over 200 μg/mL [Figure 1a], suggesting that GE has a modest potency toward HER2-overexpressing breast cancer cells. HER2-overexpressing breast cancer cells are highly metastatic cells. A study of the galangal cytotoxicity on another highly metastatic cells reported that the IC_{50} of GE in TNBC 4T1 cells was 135 μg/mL. Moreover, GE was not cytotoxic in a normal fibroblast NIH 3T3 cell line.[15] This result suggests that GE is selective and relatively safe toward healthy cells.

The modest cytotoxicity of GE reflected in the apoptosis analysis, which showed that GE did not induce apoptosis [Figure 2]. However, in the cell cycle analysis, we observed that GE treatment caused cell arrest at the G2/M phase [Figure 1c]. Therefore, we thought that another mechanism might be attributed to the decreased cell viability under the treatment of GE. In this study, we investigated the possibility of senescence involvement. Our results showed that GE-induced cell senescence comparable with that under Dox [Figure 3]. Various extrinsic factors, including conventional chemotherapeutic agents, oxidative stress, and genetic manipulations, could trigger senescence. This form of senescence termed as stress-induced premature senescence,[6] and it can be induced in tumor cells.[22]

Concerning this finding, we examined the GE effect on the intracellular ROS level. As expected, GE treatment caused elevated ROS levels comparable with Dox treatment [Figure 4a and b]. These findings raise the potential of GEs utilization as a supportive agent to treat HER2-overexpressing breast cancer cells. One primary function of HER2 is to increase cell survival by inhibiting apoptosis that leads to cell overproliferation and tumor growth.[23] The Dox and GE treatments at the concentrations used and designated incubation time were insufficient to direct this type of cells to undergo apoptosis [Figure 2]. However, by targeting senescence, these cells stopped propagating. A strategy to target senescence maybe is more effective and practical than the previous approach, which targets apoptosis.[18] Our result in Figure 4c indicated that the combination of Dox and GE decreased cell viability more than the Dox treatment alone. This result confirms the previous results shown in Figures 3a, b, and 4b, suggesting that the decreased cell viability was due to elevated cell senescence triggered by increased intracellular ROS level under the combined treatment by Dox and GE. Senescence cells may be detected as viable cells in cytotoxicity assay, although the cells undergo growth arrest. A similar result also observed in TNBC and 4T1 cells.[15] The 4T1 cells treated with 50 μg/mL GE were at the G2/M phase, and their numbers increased when the GE treatment administered at 100 μg/mL. Although the apoptosis assay showed no remarkable increase in the apoptosis event, the senescence assay showed that the treatment of GE increased the cellular senescence of 4T1 cells.

As we mentioned before, oxidative stress, including ROS, could trigger senescence. The ROS level plays an important role in the metabolism dysregulation of cancer cells. The rate of proliferation within cancer cells is considerably higher than that of healthy cells, resulting in the former's notably higher ROS level. Thus, the ROS level in cancer cells is at a high steady state.[24] The interference with pro-oxidizing and antioxidant agents suggested causing highly oxidative stress and cytotoxicity. Such a condition occurs because at high ROS levels, cell division halted, and cells undergo cell cycle arrest and eventually die from apoptosis.[25] We have described in the previous paragraph that in our study, the induction of cell senescence by GE involved the elevated intracellular ROS. ACA, the bioactive compound in *A. galanga* rhizomes and seeds, increases the intracellular ROS level in HepG2 human hepatocellular carcinoma cells.[26] Moreover, ROS increase involves the ROS-generated NADPH oxidase activity, which increased on treatment of ACA but did not affect the activities of oxide-scavenging enzymes (catalase and glutathione peroxidase). In the form of crude extract, galangal also increased the intracellular ROS level in 4T1 TNBC cells.[15] However, the effect of GE in inducing the ROS level may not rely only on ACA. 4'-HCA, which also extracted from *A. galanga,* induced the ROS production in HL-60 and U937 cells.[13] Interestingly, the GE effect related to cancer metabolism caused no effect on normal cells, including hepatocytes[26] and fibroblast cells (NIH3T3),[15] indicating that GE selectively interferes with cancer metabolism by shifting the redox balance in cancer cells to become more pro-oxidant. Thus, GE increases the oxidative stress level to surpass the toxicity threshold and only affects the cancer cells with a higher baseline intracellular ROS level than the healthy cells.

The effect of natural compounds on interfering with cancer metabolism is not only found in GE. Other groups also reported a similar effect from other natural compounds. Resveratrol reported exerting anticancer and chemoprevention activity by inducing premature senescence in lung cancer. The low dose of resveratrol induces significant cellular senescent, and this condition correlates with an increased level of p53 and p21.[27] The cytotoxicity of curcumin in erythroleukemia cells involves its ability to directly bind various ROS metabolic enzymes, elevating the ROS level.[20] As reviewed by Meiyanto and Larasati, brucein D and xanthorrizol also exhibit pro-oxidative activity and cause cytotoxicity toward pancreatic cancer and squamous cell carcinoma cells, respectively.[28] Therefore, it is interesting to further explore the activity of GE and its active compounds in cancer metabolism as a target to inhibit cancer cell proliferation.

CONCLUSION

Altogether, GE showed the capability to halt HER2-overexpressing breast cancer cell proliferation, and this was associated with the induction of cellular senescence. Although further investigation is still needed to clarify the mechanism of GEs senescence induction, our study indicated that it might involve the ability of GE to increase cellular ROS level.

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CONFLICTS OF INTEREST

We declare that we have no conflicts of interest.

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