Inhibition of breast cancer cell development using *Citrus maxima* extract through increasing levels of Reactive Oxygen Species (ROS)

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Abstract. Excessive Reactive oxygen species (ROS) level can lead to the activation of apoptosis and inhibit metastasis through the NF-κB pathway. Therapeutic approaches by increasing ROS level may be effective strategic in cancer treatment. *Citrus maxima* (CM) is one of a natural medicinal plant that exhibits cytotoxic effect on several cancer cells. However, the CM extract effect to regulate ROS level remains unclear. Therefore, in this study aims to evaluated the cytotoxic effects of CM extract on metastasis breast cancer MDA-MB-231 cells. Cytotoxic activity of CM extract was evaluated using MTT assay, ROS levels was calculated under flowcytometry. Furthermore, the binding interaction between phytoconstituents and protein that responsible on the ROS metabolic pathway was analyzed under in silico molecular docking. The presence of CM extract increased cells death with IC₅₀ value of 338 μg/mL for 24 h through induction of ROS levels. Molecular docking studies revealed that the hesperidin displayed the best binding energies at -21.4766 (NF-κB) kcal/mol. The binding interaction of hesperidin with the active site of NF-κB protein suggested that amino acid residues (His537, Asp519, Gly407, Gln479, Arg416) might play a role in ROS metabolism. These findings have shown that CM extract inhibited cancer proliferation by increasing ROS levels.

1. Introduction

Breast cancer (BC) is the leading cause of cancer death worldwide among women [1]. The common cause of cancer death due to the complexity of the cancer [2]. The complexity is due to its multifaceted and multifactorial presentation. Over the last few decades, oxidation-reduction (redox) imbalance has been shown to be one of the most significant explanations for cancer growth, progression, and metastatic in human cells [3]. Anticancer therapy based on oxidative damage due to acceleration of the accumulative reactive oxygen species (ROS) or defective antioxidant mechanism in cancer cells has been developed [4]. The concept of this mechanism is the drug could elevate ROS levels over the cytotoxic threshold, accompanies with down regulation of cellular antioxidant can selectively kill cancer cells [5,6]. When an exogenous ROS-producing agent is activated, it becomes redox imbalanced. Cancer cells become more fragile than normal cells, thereby leads to cell death. On the other hand, previous
study also reported that ROS signaling molecules involved in multiple cellular signaling pathway important for the proliferation of cells. Therefore, developing methods for the appropriate use of ROS in anti-tumor applications is very challenging but worthwhile.

_Citrus maxima_ peels (CM) is waste and do not used in Indonesia. However, several studies have shown that the main component of CM has been regarded as an important source for novel compounds for the discovery of anticancer drug. Recently, CM have strong cytotoxic effect on several breast cancer cells [7–11]. A few studies have reported that CM can inhibited cancer cells growth through induction of apoptosis dan cell cycle arrest [9]. However, the mechanisms underlying it remain largely unclear. Many of the compounds and active ingredients in CM may have some anticancer effects followed by changes in cellular ROS. Therefore, this study aims to determine the cytotoxic activity of CM in human breast cancer development mediated by ROS MDA-MB-231.

2. Methods

2.1. Preparation of crude extracts of _Citrus maxima_

_Citrus maxima_ peels were collected from Semarang, Indonesia and dried. Shade-dried and powdered peels were successively extracted using ethanol. 550 g of dry peels powder mixed with ethanol 96% and extracted at room temperature for 72 h and dark condition. The macerate was filtered with Whatman filter paper and the filtrate was concentrated under reduced pressure on rotary vacuum evaporator (IKA) at 50°C. The crude extracts were dried in a vacuum freeze dryer and preserved at -20°C for subsequent analysis.

2.2. Molecular docking

The 3D structure of CM secondary metabolites was obtained from PubChem (www.pubchem.ncbi.nlm.nih.gov) and prepared using ChemAxon (www.chemaxon.com) (Figure 1). For the current investigation, the validated drug target NF-KB was imported from protein data bank (PDB) with the PDB code 4DN5. The YASARA software was used to prepared the protein before docking simulation (www.yasara.org/viewdl.htm). Further, to calculate the possible binding target of compound, various docking simulation were carried out using Autodock Vina molecular docking software. On the final step, visualization of docking simulation in this study was determine under PyMol www.pymol.org.

2.3. Cell culture

Human highly metastatic breast cancer cells (MDA-MB-231) were obtained from Stem Cell and Cancer Research (SCCR Indonesia), Semarang, Indonesia. The cells were cultivated in sterile T75 flasks in DMEM high glucose medium enrich with FBS 10% v/v, penicillin-streptomycin 1% v/v and amphotericin B 0,25% v/v. Cells were grown in monolayer culture at 37°C in humidified air containing 5% CO₂.

2.4. Cell viability assay

The cell viability test of the CM toward MDA-MB-231 cells were analysed under MTT assay [12,13]. Briefly, 5x10³ cells/well suspended in 100 µL DMEM complete medium were seeded into 96-well plate. After 24 h incubation at 37°C in 5% CO₂ the several concentrations of CM (10-400 µg/mL) were added in triplicate and the cells were further incubate for 24 h. After that, the medium of each well has been replaced by DMEM containing 0.5 mg/mL of MTT and incubated at 37°C for 2-4 h. The formazan crystals were dissolved in DMSO and the absorbance was assessed 595 nm. The percentage of cell viability was calculated as Y= BX + A. The IC50 values were calculated using Microsoft Excel version 2010 software. Negative controls were maintained with DMSO [14].

2.5. Measurement of ROS intracellular
The production of intracellular ROS was calculated using a fluorometric assay with the cell-permeable reagent 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as mentioned above [15]. Briefly, 2 x 10^5 cells/well MDA-MB-231 cells were seeded in six-well plates and incubated at 37°C overnight. After 24 h, the medium was removed and each well was washed repeatedly with 500 µL of saline phosphate buffer (PBS). Then, DCFH-DA (10 µM) were applied into each well and incubated at 37°C for 30 min in the dark. The solutions were separated and each well flushed with 200 µL of PBS. CM at several concentration (30µg/mL and 300µg/mL) and doxorubicin 10 nM as positive control were introduced to cells. Fluorescence intensity was assessed under flowcytometry (C6 Flow cytometer, BD Biosiences, CA) after 30 min incubation at 37°C. Fluorescence emission of each well was measured prior to the addition of DCFH-DA and was considered as background. The ROS level was measured as a percentage of the control wells after background subtraction for each well.

2.6. Statistical analysis
The data was presented as the mean ± SE and statistical analyses were performed using SPSS 24. The significance of the differences between the two experimental conditions was tested using ANOVA. Values for the variations have been applied to each statistic.

3. Results and Discussion
In this study, CM has shown excellent anticancer effects in metastatic breast cancer cells by increasing ROS intracellular levels. These findings suggested that CM should be further investigated as an effective natural anticancer agent for the treatment of breast cancer. Accumulating evidence indicates that increasing oxidative stress can be an effective strategy for killing cancer cells [4]. Agents with the ability to induce ROS levels have anticancer effects in breast cancer cells.

![Figure 1](image1.png)

**Figure 1.** (A) The concentration-response curve of MDA-MB-231 cancer cells derived from MTT cytotoxic assay performed after 24 h exposures with CM. Data are presented as mean ± SE (n=3). (B) Morphological changes of MDA-MB-231 cells were determine under inverted microscope at 100x magnification. Black arrow: cell shrinkage; red arrow: cell rounding and detachment; blue arrow: membrane blebbing. Experiment is completed in three independent experiment in triplicate.

To assess the cytotoxic effect of CM on MDA-MB-23, an MTT assay was performed. CM significantly decreased the viability of MDA-MB-231 cells in a dose-dependent manner. The IC_{50} value
of CM was 326 µg/mL for 24 h (Figure 1A). In addition, the CM caused morphological changes under inverted microscope observation (Figure 1B). The high concentration of CM induced cell shrinkage, pyknosis and fragmentation. Cell shrinkage, cell detachment, cell rounding and membrane blebbing are visible through inverted microscopy during the early process of apoptosis [16]. The results can be closely related with previously reported secondary metabolite compound isolated from CM. Previous studies have identified the presence of hesperidin potentially inhibit the growth of BC [17–19]. In addition, hesperetin inhibited cancer cells growth on HER2+ breast cancer cells through induction cell cycle and apoptosis [20]. These findings clearly indicated that CM has a potential reduce cell viability of MDA-MB-231 breast cancer cells may be through apoptosis induction. The one of apoptosis pathway is by inducing generation of ROS. The excessive ROS level can lead to the activation of apoptosis and autophagic cell death through the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [21].

Furthermore, to explore the mechanism of cells death we explored whether intracellular ROS levels was involved in the anti-cancer effects of CM. The ROS levels was analyzed using the DCFH-DA fluorescent probe that recorded H2O2. The CM treatment at low concentration 30 µg/mL significantly decreased ROS level compare with untreated. Interestingly, the high level of CM 300 µg/mL dramatically increased ROS level up to 17-fold compare to untreated (Fig 2A-B). In addition, CM also induced higher ROS level than doxorubicin (DOX) as a positive control. Collectively, these findings suggested that ROS generation plays a central role in mediation of CM-inhibited cell growth. These data supported the previously study that reported the nobiletin, a secondary metabolite compound of CM reduced cells growth through ROS induction [22,23]. The cytotoxic ROS signaling appears to be regulated in part by activation of the c-Jun-N-terminal kinase mitogen-activated protein kinase cascade, while in some systems ROS lead to activation of NF-kB [24]. The activation of NF-kB is a crucial role in apoptosis, proliferation and induction epithelial mesenchymal transition (EMT) [25]. EMT is a main marker of cancer leading metastasis. So, it is necessary to investigate the effects of CM compound on the NF-kB pathway. The binding interaction of several secondary metabolite of CM with NF-kB protein was performed using in silico approach.

Figure 2. (A) ROS-FITC profile (B) Cell Counts MDA-MB-231 cells without treatment, after DOX treatment, CM 30 µg/mL, and CM 300 µg/mL (B). DOX were carried out for 24 hours shows to be able to significantly increase intracellular ROS levels compared to a single DOX treatment.
The docking score of hesperidin to NF-kB protein showed less than native ligand. The docking score of hesperidin and native ligand was -21.4766 kcal/mol and -10.1817 kcal/mol, respectively (Figure 3). The phenomenon suggest that hesperidin performs better interaction to the NF-kB protein compared to native ligand. On the NF-kB, hesperidin interacted with the amino acid residues Phe411, Gly402, Lys429, Leu522, Leu 406, Leu472, Glu 470, Gln478, Val414, Arg416, Gly40, Asp519, and His 517. Overall, the CM may have had possible interaction capabilities with NF-kB. In conclusion, our studies indicate that CM can inhibit breast cancer cells proliferation through increased level of ROS. We have further shown the mechanism by which to kill cancer through ROS by activating NF-kB. Hesperidin from CM has a better interaction than the native ligand. More importantly, our results further indicate that developing agents with inducing ROS potential will be a good strategy for cancer therapy.

![Figure 3](image-url)  
**Figure 3.** Binding interaction profile of hesperidin compared to native ligand.

4. Conclusion  
The data suggest a novel mechanism by which *Citrus maxima* inhibits breast cancer cell growth through the formation of excessive ROS.

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6

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