



Citrus sinensis (L) Peels Extract Inhibits Metastasis of Breast Cancer Cells by Targeting the Downregulation Matrix Metalloproteinases-9

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Abstract

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BACKGROUND: Long-term use of doxorubicin (DOX) chemotherapy causes several side effects, especially induction of metastasis on breast cancer (BC). There is an urgent need to identify novel agent with low side effect targeting BC metastasis. *Citrus sinensis* (L.) peels extract (CSP) has long been used for the treatment of several cancers. However, its anti-metastatic potential against BC metastatic remains unclear.

AIM: This study aimed to explore the role of CSP in combination with DOX in inhibiting the migration of metastatic BC MDA-MB-231 cells.

MATERIALS AND METHODS: Potential cytotoxic in single and combination was analyzed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay [MTT] assay). The anti-metastatic activities of several major compounds on CSP including hesperetin, tangeretin, nobiletin, naringenin, and hesperidin were screened by molecular docking under protein-ligand ant system software.

RESULTS: Based on molecular docking, we revealed that the selected protein target matrix metalloproteinases-9 (PBD ID:2OVX) has lower docking score for hesperetin, tangeretin, nobiletin, naringenin, and hesperidin compare to DOX. CSP and DOX individually exhibited strong cytotoxic effect on MDA-MB-231 cells under MTT assay with IC50 value of 344 µg/mL and 85 nM, respectively. Furthermore, CSP in combination with DOX synergistically increased the cytotoxicity of DOX. Here, we showed that CSP can specifically suppress the side effect of DOX-induced metastasis by reduces doses of DOX. However, low doses of DOX in combination with CSP still potential inhibited cancer cells growth.

CONCLUSION: CSP increased the cytotoxicity and inhibited the induction of metastasis by DOX in BC cells so that CSP potential to be developed as co-chemotherapeutic agent.

Introduction

Breast cancer (BC) is the most frequent cancer among women, with 3.8 million death case in 2019 [1]. The most cases of BC death are mainly due to metastasis of primary cancer to various organ sites. It is a multi-faceted mechanism that results from orchestrated activities, including adhesion, invasion, and migration [2]. Extracellular matrix degradation (ECM) by proteolytic enzymes and subsequent cancer invasion is important early stages of metastasis [3]. Matrix metalloproteinases (MMPs) is the most crucial proteolytic enzyme that degrades the ECM. Accordingly, the MMP-9 expression is associated with BC metastatic and invasion [4], [5]. Therefore, it is necessary to develop new strategy that has the potential to inhibit metastatic BC cells.

Recently, the main treatment for BC metastatic is chemotherapy [6]. However, this therapy is not effective and often leads to side effect such as metastatic and relapse [7]. The

previous study reported that doxorubicin (DOX) induces lamellipodia, this formation is the initial stage of metastatic [8], [9]. Thus, studies toward finding efficient therapeutic strategies with minimum side effects are critical for expansion of existing treatment options for BC. Natural herbal medicine is rich sources of bioactive molecules and being developed to compensate for the drawbacks and toxicity of chemotherapy. Flavonoid from *Citrus sinensis* proven to have an anticancer activity with multiple target side on several type of cancer. Hesperetin, a secondary metabolite of *C. sinensis* inhibited Rac-1 expression, the expression of a gene that regulated metastatic [10]. Hesperidin is also shown to be inhibited MCF-7 cancer cell grow [11]. However, the mechanism of *C. sinensis* for inhibiting DOX-induced metastasis is remain unclear. In this study, we investigated the effect of *C. sinensis* peels extract (CSP) in combination with DOX on BC metastatic MDA-MB-231 proliferation and its molecular mechanisms of action through *in silico* approach.

Materials and Methods

Plant material

C. sinensis (Rutaceae) peels were collected from Bandungan, Semarang – Central Java, Indonesia. The identity of the plant was confirmed by biologist at Biology Department, Diponegoro University.

Preparation of plant extract

The peels of *C. sinensis* was collected, chopped into small pieces and dried under shade. The dried peels (250 g) were re-macerated using ethanol 96% for 3 days at room temperature based on [12] with slight modification. The extracts were evaporated in the under reduce pressure in a rotary vacuum evaporator at 50°C to result the crude extracts. The crude extracts were dried in a vacuum freeze dryer and preserved at -20°C for subsequent analysis.

Cell culture

MDAMB-231 (ECACC #92020424) were cultured in DMEM medium (Gibco, San Francisco, CA, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 12.5 µg/ml Amphotericin B (Gibco, USA), 150 µg/ml Streptomycin, and 150 IU/ml Penicillin (Gibco, USA). The cell culture was cultivated at 37°C under 5% CO₂. Culture media were renewed every 2–3 days, and cells were subculture when confluent of 80–90%. For assays, only cells with >90% viability, passage number <10, and in the log growth phase were used.

Cell viability assay

The cell viability was based on a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay according to Mosmann [13], Suzery *et al.* [14] with slight modification. MDA-MB-231 5×10^3 cells/well was seeded in 96 well-plate and incubated at 37°C under 5% CO₂ for 24 h. Cells were treated in several concentration of CSP (10–500 µg/ml), DOX (1–500 nM) or CSP/DOX combination exposed for 24 h. Untreated cells were regarded as negative controls. After treatment, cells were treated with 0.5 mg/mL of MTT (Biovision) and incubated further for 4 h. Then, DMSO 100 µl was added to dissolve the crystal of formazan. The absorbance was measured by ELISA reader (Biorad iMark™ Microplate Reader) at λ 595 nm [14].

Combination index (CI)

The combined effect of CSP and DOX was evaluated using CI and isobologram were analyzed with CompuSyn® software based on the Chou-Talalay

method as describe previously [15]. The combination effect is defined as: CI <1 is a synergistic effect, CI = 1 is an additive effect, and CI >1 is an antagonistic effect.

Molecular docking

The protein-Ligand Ant System program was used for simulation of molecular docking. The structure of protein MMP-9 (PDB ID: 2OVX) has been download from www.rcsb.org The YASARA software was used to prepared the protein before docking simulation (www.yasara.org/viewdl.htm), on the other hand, the ligand was prepared using ChemAxon (www.chemaxon.com/marvin/download-user.html). Furthermore, the visualization of docking simulation in this study was determined under PyMol www.pymol.org.

Statistical analysis

Data are presented as the means ± standard deviation. All calculations were carried out using IBM SPSS 22.0 (IBM Corp., Armonk, NY, USA). The statistical significance of the differences group's differences was assessed using one-way ANOVA and continued with Fisher's least significant difference *post hoc*. $p < 0.05$ was considered significant.

Results

The effect of CSP and DOX on MDA-MB-231 cell viability

A single treatment of CSP and DOX individually possessed strong cytotoxic effect with dose-dependent manner. After 24 h treatment, the IC₅₀ value of CSP and DOX in MDA-MD-231 was 344 µg/mL and 85 nM, respectively (Figure 1). Interestingly, low doses of DOX changed cell morphology, which is characterized by elongation of lamellipodia (Figure 2).

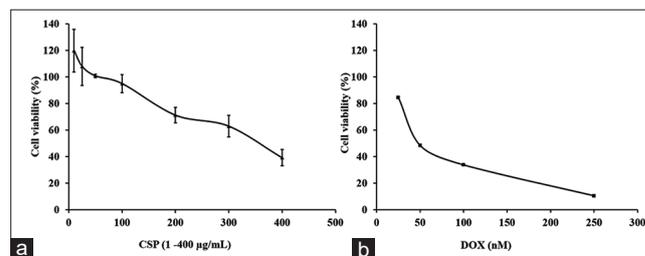


Figure 1. : Cytotoxic activity of *Citrus sinensis* peels extract (CSP) and doxorubicin (DOX) on MDA-MB-231 cells. Cells 5×10^3 were seeded for 24 h in 96 -well plate, then treated with (a) CSP and (b) DOX individually and incubated for 24 h. cell viability profile expressed mean ± SE of 3 experiments. IC₅₀ obtained from a linear regression calculation of log concentration versus cell viability with $p < 0.05$.

In this study, CSP has been explored as natural co-chemotherapeutic agent against highly

metastatic BC cells. To explore the combination effect of CSP and DOX on cell viability, we used one-eighth, one-fourth, and half of IC_{50} value of CSP and DOX. The combination of 177 $\mu\text{g}/\text{mL}$ of CSP and 42.5 nM DOX decreased cell viability up to 5.11% (Figure 3a). Based on the cell viability on combination treatment, we calculated the CI using Chou-Talay methods. A CI plot showed that all off combination treatment exhibited a synergistic effect with CI value 0.55–3.48 (Figure 3b-c). Seven plot indicated antagonistic effect and nine plot indicated synergistic effect. The highest synergistic effect was CSP one-sixteenth IC_{50} and DOX one-fourth

IC_{50} . The isobologram showed that isobologram plot of CSP/DOX combination was positioned to the bottom left of the curve, which suggested that CSP had a synergistic effect with DOX.

Molecular docking study of secondary metabolite of CSP on MMP-9

The CSP contains various metabolite compound that possess cytotoxic activity on several cancer cells, such as hesperetin, hesperidin, naringenin,

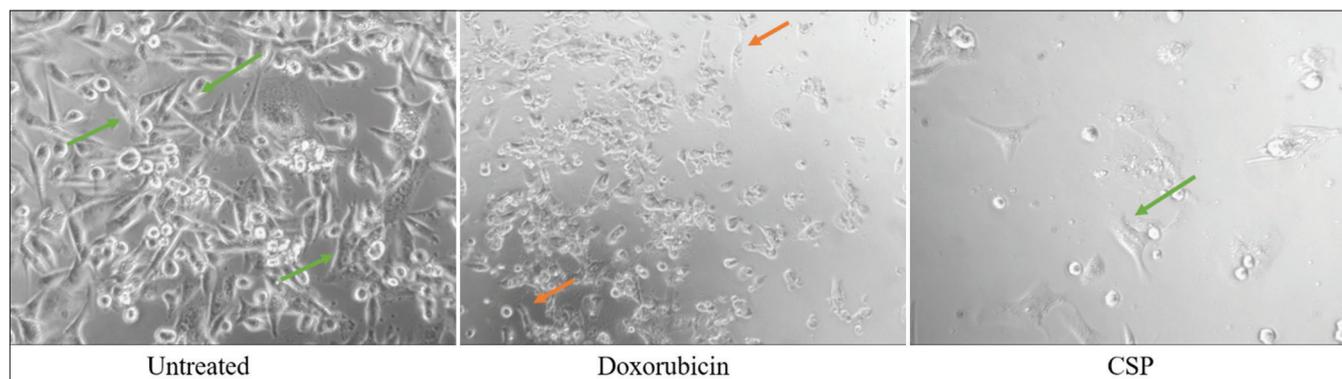


Figure 2: The effect of Citrus sinensis peels extract (CSP) and doxorubicin (DOX) on cell morphology. Visible morphological changes and population of cells in treatment of untreated, DOX 10 nM and CSP 200 $\mu\text{g}/\text{mL}$. Observations of Cell morphology performed using an inverted microscope with a magnification of 100 \times . Green arrow indicated live cells and red arrow indicated lamellipodia elongation and shrinker cells

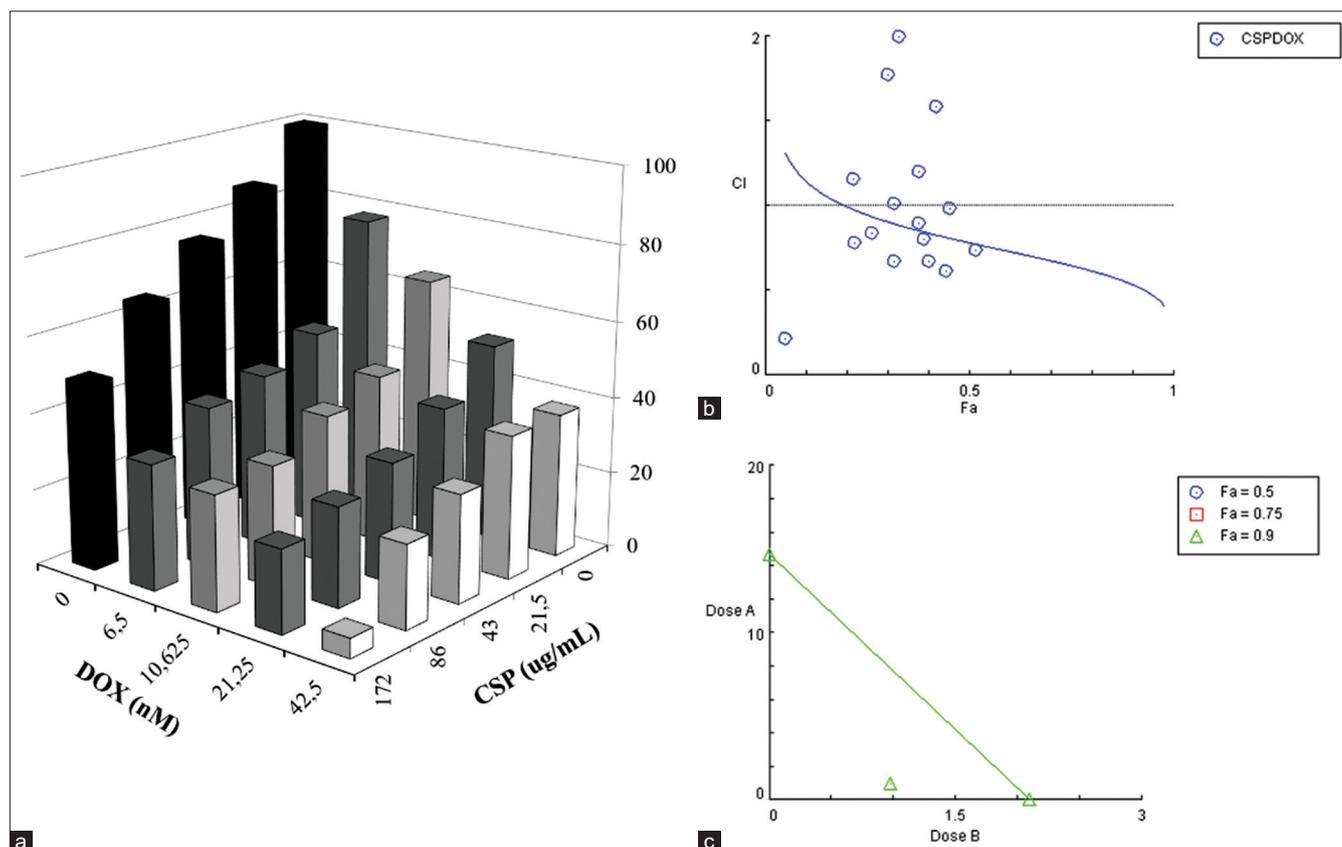


Figure 3: (a) Effect of Citrus sinensis peels extract (CSP) in combination with doxorubicin (DOX) on the MDA-MB-231 cell viability. (b) Combination index plot, and (c) isobologram for a combination of CSP and DOX calculated by CompuSyn software based on Chou-Talay method

nobiletin, and tangeretin [16], [17], [18], [19]. The evaluation of the binding activity showed that all of CSP secondary metabolite compound possessed a higher docking score than that DOX. On the other hand, according to the visualization study of CSP secondary metabolite compound performed different binding interaction compared with those of DOX and native ligand (Table 1 and Figure 4). Hesperetin has the higher binding interaction with MMP-9 protein. On the MMP-9, hesperetin interacted with the amino acid residues Tyr 423, Leu 418, Leu 188, Ala 189, Gln 402, and His 411.

Discussion

The MTT assay was used to evaluate the cytotoxic effect to determine the potency of CSP to enhance the cytotoxicity of DOX on highly metastatic BC MDA-MB-231 cells. The aims of this study were to develop CSP as a co-chemotherapy agent to inhibit DOX-induced metastasis. The point of co-chemotherapy is to decrease the side effect and enhance the efficacy of drug [20]. CSP is a natural herbal medicine which

proven to have a cytotoxic effect on several cancer cells. Hesperetin, a one of secondary metabolite of CSP show to enhance the effect of DOX against HER2+ BC cells [10]. Hesperidin is also enhanced the effectiveness of DOX and 5-FU through apoptosis induction [11], [21]. A single treatment of CSP and DOX individually possessed strong cytotoxic effect with dose-dependent manner and the low dose of DOX induced lamellipodia elongation. This phenomenon supports the previous studies that DOX in concentration of 10 nM induced lamellipodia formation through the activate transforming growth factor beta (TGF β) signaling pathway [9], [22]. The activation of TGF β induces EMT caused inducing motility of cell and metastatic [8]. Further, the cytotoxic combination showed that that CSP had a synergistic effect with DOX with CI value <1. These results suggested that CSP could sensitize highly metastatic BC MDA-MB-231 cells to DOX. Therefore, the further assay was need to be evaluated the molecular mechanism of CSP secondary metabolite compound on the MMP-9 interaction through molecular docking approach.

The CSP contains various metabolite compound that possess cytotoxic activity on several cancer cells, such as hesperetin, hesperidin, naringenin, nobiletin, and tangeretin [16], [17], [18], [19], [23]. A simulated interaction analysis of the CSP secondary metabolite compound was performed in the target sample using molecular docking simulation to validate the CSP molecular mechanism of the antimetastatic behavior hypothesis. The target protein for screening the antimetastatic effect was MMP-9. The MMP-9 protein, which played a role in ECM, was used as a target protein for antimetastatic activity screening [24]. Statistical

Table 1: Docking score of secondary metabolites CSP on MMP-9

Ligand	Binding interaction (kcal/mol)	RMSD (Å)
Native ligand	-37.4444	0.3603
DOX	-22.4682	1.9025
Hesperidin	-23.9460	1.4127
Hesperetin	-25.0878	1.3572
Naringenin	-22.5614	1.4914
Nobiletin	-24.0417	1.2355
Tangeretin	-25.0748	1.7595

DOX: Doxorubicin, MMP-9: Matrix metalloproteinases-9.

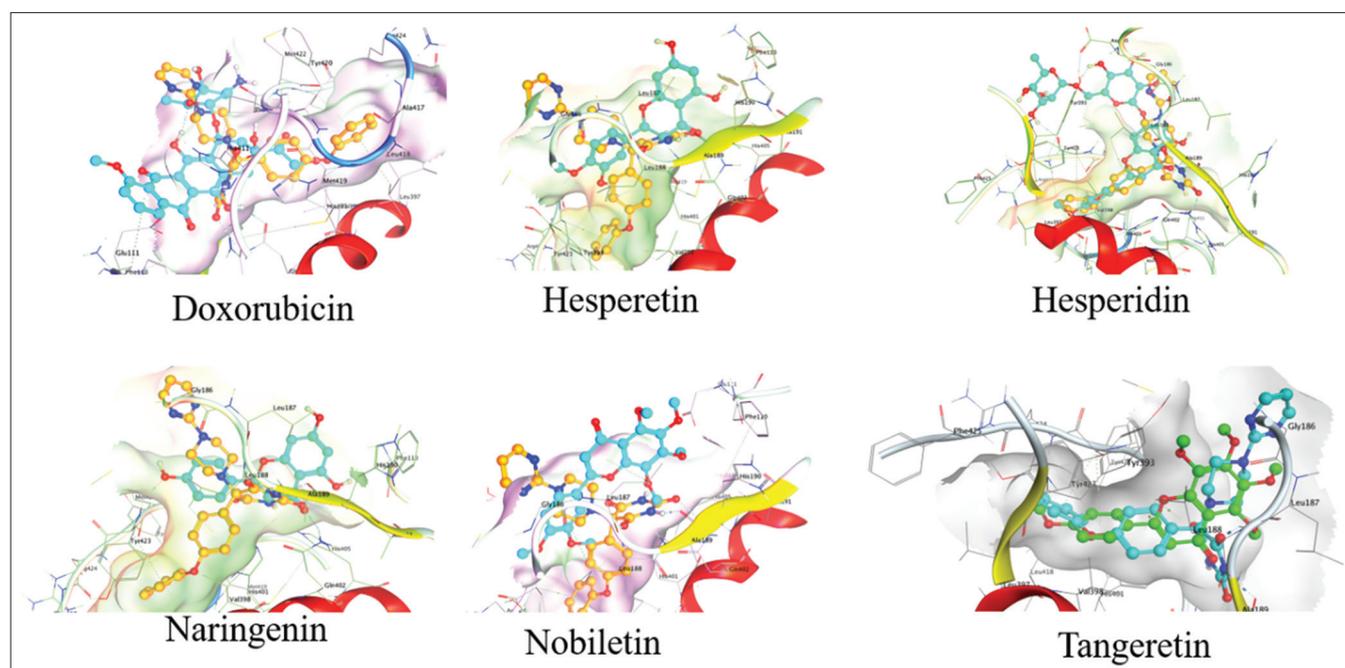


Figure 4: The visualization of compound interaction on matrix metalloproteinases-9. Molecular interaction was evaluated using PyMol. Compound is represented as gold/green balls and sticks, while the native ligand is represented as Tosca balls and sticks

analysis showed that the molecular docking findings were valid based on the RMSD value of $<2 \text{ \AA}$ (Table 1). The evaluation of the binding activity showed that all off CSP secondary metabolite compound possessed a higher docking score than that DOX. Overall, the CSP may have had possible interaction capabilities with MMP-9. Thus, CSP is a potential candidate that can be used as a natural co-chemotherapeutic agent based on its antimetastatic activity.

Conclusion

The combination of CSP and DOX enhanced sensitivity of MDA-MB-231 BC cells toward DOX. Furthermore, CSP metastasis cell through MMP-9 interaction. More importantly, our findings suggested that CSP potential to be developed as natural cancer therapeutic agent.

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