Journal of Tropical Biodiversity and Biotechnology
Volume 10, Issue 04 (2025): jtbb18058
DOI: 10.22146/jtbb.18058

Research Article

The *Citrus sinensis* Peel Extract Elevates the Cytotoxicity Effect of Doxorubicin and Inhibits 4T1 Cell Migration

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Keywords:
Citrus sinensis
Co-chemotherapy
Doxorubicin
Sinensetin
TNBC
Submitted:
03 December 2024
Accepted:
27 April 2025
Published:
17 November 2025
Editors:
Ardaning Nuriliani
Annisaa Widyasari

ABSTRACT

Combination therapy using natural-based substances is an effective approach to increase the efficacy of doxorubicin (Dox) while reducing its toxicity towards normal cells. This general chemotherapeutic agent treats various cancer cells, including triple-negative breast cancer (TNBC). *Citrus sinensis* peel extract (CPE) contains sinensetin (Sin), which exhibits anti-cancer effects, offering good prospects as a co-chemotherapy agent. This research aims to evaluate the co-chemotherapeutic potency of CPE-contained Sin when combined with Dox and also study its influence on 4T1 cell migration. Assays performed in this experiment included cell viability assay using MTT in individual and combination manner, clonogenic assay to analyse the proliferation inhibition effects of the treatments, scratch wound healing assay to assess the cells' migration status, and gelatine zymography to evaluate cells' matrix metalloproteinase-9 (MMP-9) activity. This experiment confirmed that CPE and Sin had cytotoxic effects on 4T1 cells with the IC_{50} of 536 µg mL^{-1} and 120 µM, respectively, while Dox performed cytotoxicity on 4T1 cells with an IC_{50} of 2 µM. The combination of CPE and Dox showed synergistic effects with the combination index (CI) <1.0. CPE and its co-administration with Dox permanently suppressed the colony formation of 4T1 cells after 10 days of treatment removal. CPE and Sin inhibited the 4T1 cell migration strongly compared to Dox. CPE in its single and mixture form with Dox inhibited the MMP-9 activity. These results suggest that CPE, when combined with Dox, could be a promising co-chemotherapy regimen for TNBC treatment, potentially reducing the risk of metastasis and relapse.

How to cite:
Zufairo', S.K. et al., 2025. The *Citrus sinensis* Peel Extract Elevates the Cytotoxicity Effect of Doxorubicin and Inhibits 4T1 Cell Migration. *Journal of Tropical Biodiversity and Biotechnology*, 10(4), jtbb18058. doi: 10.22146/jtbb.18058

INTRODUCTION

Triple-negative breast cancer (TNBC) is known for progesterone receptor (PR), oestrogen receptor (ER), and human epidermal growth factor receptor 2 (HER-2) negativity, which makes anti-oestrogen based-chemotherapeutic agents ineffective in treating this type of cancer (Manjunath & Choudhary 2021). TNBC comprises 10-15 % of all breast cancer incidence and is responsible for 40 % of the mortality caused by breast cancer worldwide (Rosińska et al. 2024). Patients with TNBC often have a poor prognosis due to the aggressiveness of the disease, such as the high rate of proliferation, metastases, and relapse (Vuger et al. 2020). Chemotherapeutic agents that are neither hormonal nor HER-2 targeted-based are needed to treat this type of cancer.

Some chemotherapy agents have been used to treat TNBC. Doxorubicin (Dox) is an oncological agent generally used to treat diverse cancer types, including TNBC (Amalina et al. 2023). Dox is a non-anti-estrogenic chemotherapy agent that induces DNA damage or double-strand breaks (DSB) in cancer cells due to the intercalation process of Dox molecules in their DNA, leading them to apoptosis (Kciuk et al. 2023). However, the use of Dox still faces some challenges, which are related to cancer cells' resistance, relapse, and migration (Wardhani et al. 2021; Kciuk et al. 2023). Therefore, there is a need for a co-chemotherapy agent to enhance the effectiveness of chemotherapy regimens, such as DOX, in treating cancer cells, especially in TNBC.

Citrus sinensis, a commercially available fruit, is known for its anti-cancer properties (Chu et al. 2017). The *Citrus sinensis* peel extract (CPE) contains several beneficial compounds that could act as anti-cancer agents. Some of these compounds are flavonoids, including sinensetin, hesperidin, hesperetin, and naringenin (Meiyanto et al. 2012; Zufairo' et al. 2023). Sinensetin (Sin) is a polymethoxy flavone that comprises as much as 1.083 % of 120 mg *Citrus sinensis* peel extract (Xu et al. 2019). It has been studied for its anti-cancer potential in several cancer cells. Previous studies have shown that Sin can reduce the viability of lung cancer cells, promote apoptosis in liver cancer cells, suppress metastasis of luminal breast cancer, and inhibit the inflammation caused by cisplatin without affecting the cisplatin's efficacy (Samidurai et al. 2020; Indriyanti et al. 2023; Li et al. 2023; Zhu et al. 2024). These promising features make CPE with Sin in it a potential co-chemotherapy agent that can be combined with Dox, enhancing its effectiveness in restraining cell progression, particularly in TNBC cells.

This study focused on assessing the effects of CPE combined with Dox on TNBC progression, particularly regarding proliferation, relapse, and its potential to inhibit cell migration. 4T1 cells were used for the TNBC model. Some assays used to assess parameters regarding proliferation, relapse, and migration were cell viability assay using MTT, colony formation assay, scratch wound healing assay, and gelatine zymography respectively.

MATERIALS AND METHODS

Materials

Citrus sinensis peel was retrieved from the local fruit market in Sleman, Yogyakarta, and determined at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada. Doxorubicin hydrochloride (Dox) was purchased from Wako, Japan (Cat. No. 046-21523). Sinensetin (Sin) was obtained from Sigma, USA (Cat. No. SML 1787-5MG). Each substance for treatment, including the extract, was dissolved in DMSO (Sigma, USA) to prepare a stock solution. These stocks were then diluted into several concentrations using complete medium for treatment purposes.

Methods

Citrus sinensis Peel Preparation

The *Citrus sinensis* peel was cleaned and dried using an oven at the tempera-

ture 60 °C for six hours, as previously described (Zufairo' et al. 2023). Dried *Citrus sinensis* was ground, sifted, and homogenised overnight using 70 % ethanol with a ratio of 1:10. The centrifugation process was conducted to distinguish the pellet and supernatant. The supernatant was collected and dried until a brown viscous extract was obtained. This extract was labelled as *Citrus sinensis* peel extract (CPE).

Cell Culture

The Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy of Universitas Gadjah Mada, kindly provided 4T1 cells. Cells were grown in Dulbecco's modified Eagle's medium (Gibco, USA). 10 % FBS (Gibco, USA) and 1 % penicillin-streptomycin (Gibco, USA) were mixed into the medium. Cells were incubated under standard conditions, which were 37 °C and 5 % CO_2.

Cell Viability Assay

4T1 cells were cultured into a 96-well plate at approximately 5 x 10^3 cells/well, incubated under standard conditions for 24h, and treated with a serial concentration of CPE (10-500 µg mL^{-1}), Sin (10-500 µM) (Sigma, USA), and Dox (0.1-10 µM) (Wako, Japan) in single and combination way (with the concentration of $^1/_2$, $^1/_4$, and $^1/_8$ of IC_{50} for each compound). All treatments were done in triplicate. Treated cells were washed with PBS 1X and were given 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, USA). Cells incubated with MTT (0.5 mg mL^{-1} in complete medium) were then treated with 10% SDS (in 0.01 M HCl) (Sigma, USA) to stop the reaction. The cells were then incubated under dark conditions overnight to be analysed using a microplate reader at 595 nm. As previously described, absorbance data were used to calculated cell viability and IC_{50} (Haryanti et al. 2022). The combination index (CI) was generated by calculating cell viability in combination treatment (Reynolds & Maurer et al. 2005).

Clonogenic Assay

The 4T1 cells were cultured into a 6-well plate (5 x 10^3 cells/well) and incubated for 24 h under standard conditions. The cells were subjected to a single and combination dose of CPE and Dox and incubated for 24 hours. The treated medium on the cells was then replaced by a fresh and complete medium and maintained for 10 days under standard conditions. All treatments were performed in triplicates. Cells were exposed to PBS 1X for rinsing, treated with formalin 10 % (Sigma, USA) for fixation, and stained using gentian violet 1 % (Sigma, USA). Dyed cells were documented and counted to analyse the colony formation of treated cells compared to the untreated ones. The ImageJ was used to analyse and count colonies from images of treated and untreated cells. The number of colonies formed was presented as % colony compared to untreated graphics.

Migration Assay

A cell suspension containing 4T1 cells (approximately 8 × 10^4 cells/well) was added to a 24-well plate and incubated for 24 h. Cells were then given a starvation medium for 24 h, which contained 0.5 % FBS. Cells were wounded by performing a scratch using yellow tips, treated using CPE, Sin, and Dox in a single form, and then incubated for 24 and 48 hours. All treatments were done in triple. Afterward, scratched cells were photographed under 100x inverted-microscope magnification and analysed using ImageJ (NIH Image, USA) to measure the scratched area. The scratched area of each treatment was used to calculate % closure at 24 and 48 h after treatments. The result was analysed against the untreated cells.

MMP-9 Activity Assay

Matrix metalloproteinase-9 (MMP-9) activity was analysed via gelatine zymography. 4T1 cells with approximately 5 x 10^5 cells/well were cultivated in a 6-well plate for 24 hours. Cells were given CPE, Dox, and their combination in starvation medium (0.5 % FBS) and maintained for 24 hours. The treatment media were collected and separated using SDS-PAGE (8 % gel, 0.1 % gelatin). The marker used in this assay was PM5100 Excelband™ 3-color pre-stained protein ladder (SMOBIO, Taiwan). The gel was renatured in 2.5 % Triton X-100 (Sigma, USA) for 30 minutes, washed, and incubated for 24 hours in an incubation buffer (40 mM Tris-HCl pH 8, 10 mM $CaCl_2$, 0.02 % NaN_3). After staining with 0.05 % Coomassie Brilliant Blue G-250 (Sigma, USA) for 2 hours, the gel was destained until clear bands appeared, indicating MMP-9 gelatinolytic activity. Band intensity was quantified using ImageJ.

Statistical Analysis

All the treatments in the assays used in this work were carried out in triplicate. Data normality was confirmed using the Shapiro–Wilk test. Normal data was then analysed statistically by independent t-test using GraphPad Prism 10.1.2, which has 95 % reliability.

RESULTS AND DISCUSSION

CPE and Sin Cytotoxic Activity Towards 4T1 Cells

Cell viability assay was conducted using MTT to assess the capacity of CPE and Sin to perform their cytotoxic activity. The result revealed that CPE and Sin performed cytotoxicity effects towards 4T1 cancer cells with the IC_{50} of 536 µg mL^{-1} and 120 µM, respectively (Figure 1a and 1b). On the other hand, Dox, a general chemotherapy agent, exerted a strong cytotoxic effect on 4T1 with the IC_{50} of 2 µM (Figure 1c). Therefore, based on the IC_{50} value of each agent, it is confirmed that CPE and Sin exerted low and moderate cytotoxicity effects, respectively, whereas Dox produced a strong cytotoxic effect (Ikawati et al. 2023).

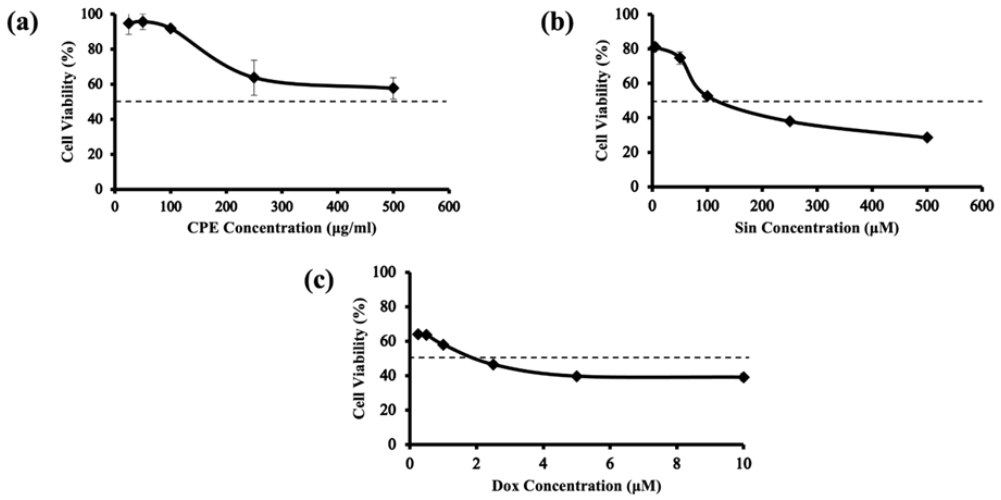


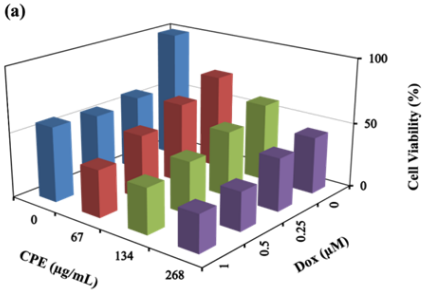
Figure 1. The cell viability status of 4T1 cells after treatment using CPE, Sin, and Dox. CPE (a) and Sin (b) have low and moderate cytotoxicity, while Dox (c) has exhibited a strong cytotoxic effect, both towards 4T1 cells. Quantitative data were presented as the mean of three independent experiments; the error bars indicate the standard error.

CPE Synergistically Enhances Dox Cytotoxic Activity on 4T1 Cells

Cell viability was known to diminish along the higher combination dose of CPE and Dox used (Figure 2a). The combination treatment of 268 µg mL^{-1} CPE with 1 µM Dox was shown to have the lowest cell viability compared to

the other dose of CPE and Dox combination. Generally, the combination index (CI) of CPE-Dox, calculated from cell viability data, exhibited a synergism on 4T1 cells. The CI within the range of 0.1-0.3 was categorised as strong synergism, 0.3-0.7 was categorised as synergism, and the range of 0.7-0.9 was categorised as moderate to slight synergism (Reynolds & Maurer 2005). All CI values for both CPE concentrations combined with various concentrations of Dox were used, which are 0.25, 0.5, and 1 µM, categorised to have strong to moderate synergistic effects with all the CI values <0.1 (Figure 2b).

(a)



(b)

CPE concentration (µg/mL)	Dox (µM)		
	0.25	0.5	1
67	0.71	0.35	0.18
134	0.38	0.28	0.26
268	0.46	0.37	0.38

Figure 2. CPE-Dox cytotoxic effect. The cell viability of 4T1 after being treated by several combinations of CPE-Dox (a) and the CI value resulting from the cell viability calculation (b) indicates the synergistic status of CPE-Dox. Each numerical data set was presented as the average of three independent experimental replicates (n=3).

CPE and Its Combination with Dox Shows Permanent Effects in the Repressing 4T1 Colony Formation

The permanent effect of treatment is expected to be exerted by chemotherapy and the candidates of co-chemotherapeutic agents to prevent the recurrence caused by the uncontrolled proliferation featured by cancer cells, especially on TNBC. A clonogenic assay was performed in this study to analyse the inhibition capacity of colony formation owned by CPE, Dox, and their combination after 10 days without treatment (Figure 3a and b). All treated cells showed the suppression of colony formation, which is very significant compared to the untreated cells. The combination treatment between CPE 250 µg mL^{-1} and Dox 1 µM performed higher inhibition than CPE and Dox in their single form. The combination of CPE and Dox generated only one small colony, while each of CPE and Dox in a single form generated two small colonies. These results indicate that CPE, in both single and combination form, showed irreversible effects in suppressing the 4T1 cell proliferation by inhibiting colony formation.

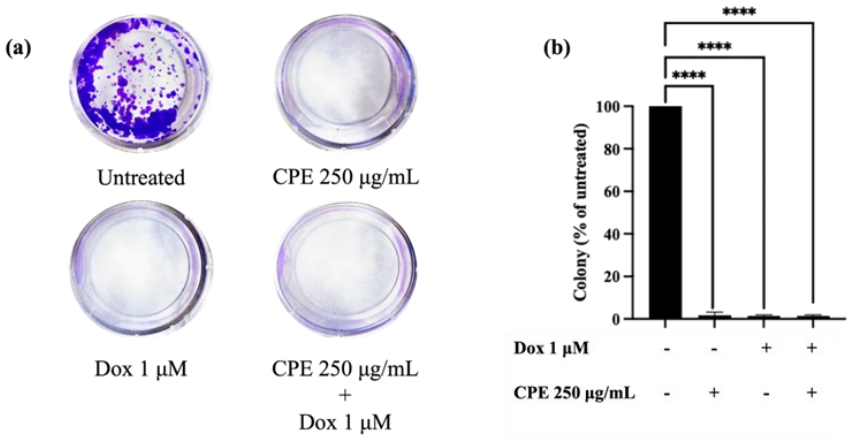


Figure 3. The colony formation of treated and untreated 4T1 cells. The appearance of colony formation of treated and untreated 4T1 cells (a), the colony formed of

treated cells presented as graphic (b). Each numerical data set reflects the average of three independent experiments. The standard deviation is represented by the error bars. ****p<0.0001.

CPE and Sin Exert Migration Inhibition Activity on 4T1 Cells

Triple-negative breast cancer (TNBC) is known as a highly metastatic type. Migration is a crucial step in the metastatic activity of cancer cells. Therefore, migration is one of several aspects that need to be targeted by chemotherapeutic and co-chemotherapeutic agents. A scratch wound healing assay was conducted in this study to evaluate the migration inhibition capacity of CPE, Sin, and Dox on 4T1 cells. Both CPE and Sin generally showed migration inhibition compared to the untreated cells (figure 4a and b). Both doses of CPE and Sin significantly inhibited 4T1 cell migration for 24 and 48 hours compared to untreated group. However, in the case of Sin treatments, the migration inhibition was stronger only with a higher dose (120 µM), even in 24 and 48 hours of treatment, which performed only 8.13 and 15.96 % of closure, respectively. In contrast, 0.5 and 1 µM Dox showed low inhibition on 4T1 cell migration compared to the untreated cells, with a % closure of 82 and 86 %, respectively, in 48 hours. Further analysis, particularly of the molecular aspects, is needed to understand the specific mechanism by which CPE, containing Sin, suppresses the migration of 4T1 cells.

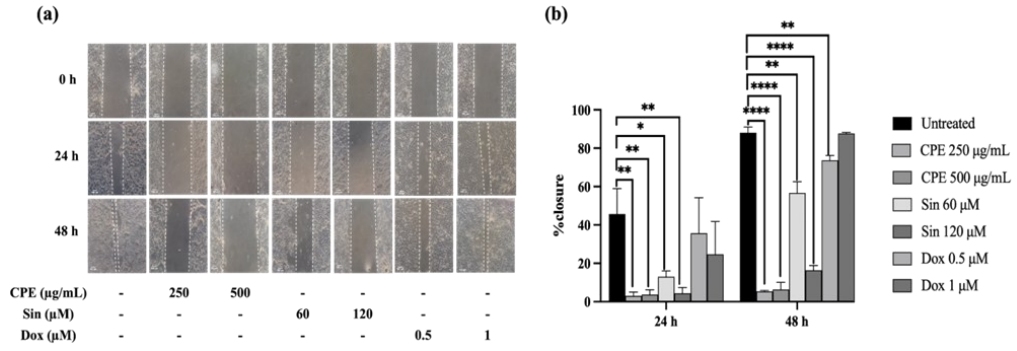


Figure 4. The migration effects of 4T1 cells after being treated by CPE, Sin, and Dox separately compared to untreated cells. The scratched area was observed on all treated and untreated 4T1 cells (a), and the graph showed the percentage of closure of each treated and untreated scratched 4T1 cell (b) from the analysis using ImageJ. Each set of quantitative data represents the average of three independent experiments, with error bars indicating the standard deviation. *p<0.05; ** p<0.01; ****p<0.0001.

CPE and Its Combination with Dox Suppress the Activity of MMP-9 on 4T1 Cells

To elucidate the specific mechanism of CPE-Dox in suppressing the migration of 4T1 cells, their effect on matrix metalloproteinase 9 (MMP-9) activity was examined using gelatine zymography. MMP-9 is a critical protein involved in the migration and metastasis of TNBC. As an enzyme, it degrades the extracellular matrix composed of collagen, facilitating cell detachment and enhancing the potential for cellular migration to other sites. The presence of clear bands in the gel was interpreted as indicative of MMP-9 activity, reflecting its ability to degrade gelatine, a collagen derivative. In this study, MMP-9 activity in 4T1 cells was found to be significantly reduced following treatment with either CPE 250 µg mL^{-1}, Dox 1 µM, or their combination. The reduction in MMP-9 activity was evidenced by the diminished intensity of the bands observed in the gel compared to untreated cells (Figure 5a) and further confirmed through quantification using ImageJ software (Figure 5b). The band intensity for CPE alone was 0.5 times lower than for Dox, with the

lowest band intensity observed in the combination of CPE with Dox. These results demonstrate that CPE, both alone and in combination with Dox, significantly reduces MMP-9 activity.

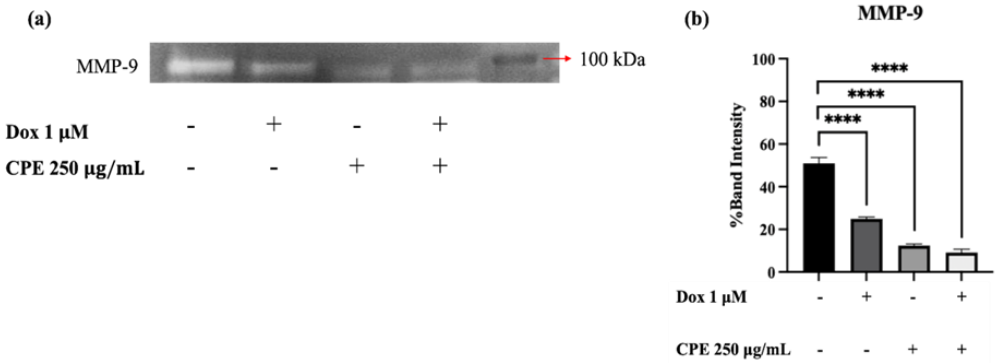


Figure 5. The activity of MMP-9 after CPE, Sin, and Dox treatments on 4T1 cells. Band which represents MMP-9 activity (a), and a graph showing percentage of band intensity from the gelatin zymography results, quantified using ImageJ (b). Each quantitative data set is displayed as the mean of triplicate experiments, with standard deviation as the error bars. ****p<0.0001.

Discussion

This work confirms that CPE enhances the cytotoxicity of Dox on the 4T1 or TNBC model. From this study, CPE demonstrated low cytotoxic effects when used alone on 4T1 cells. However, when paired with Dox in the same cell model, it shows an excellent synergistic effect. Sin, one of the polymethoxylated flavonoids in CPE, exerts a moderate cytotoxic effect on 4T1 cells. Either CPE or Sin has shown potential to increase the susceptibility of various cancer cell types to chemotherapeutic drugs, enhancing their cytotoxic effects. Previous work has shown that either CPE or Sin can enhance the cytotoxicity effects of doxorubicin and cisplatin on HepG2 (Zufairo' et al. 2023). The result of this work highlights CPE as a promising candidate for combination with other chemotherapeutic drugs.

Maintaining a persistent effect of chemotherapeutic agents is crucial to ensure their long-term efficacy and prevent cancer relapse. This study found that CPE and Dox, either alone or in combination, effectively suppressed the colony formation of 4T1 cells even 10 days after treatment cessation, demonstrating their ability to inhibit cancer cell proliferation over an extended period. CPE is known to contain various beneficial compounds, including hesperetin (Chen et al. 2012; Artanti et al. 2023). These results align with previous studies showing inhibition of colony formation in MCF-7 cells, a model of Luminal-A breast cancer, for 14 days after hesperetin treatment discontinuation (Hermawan et al. 2021). However, in this study, the compound was used as an extract at a specific concentration, and a different breast cancer cell model was employed, specifically 4T1 cells, which represent TNBC. The use of other, more human-relevant TNBC cell lines would allow for a more comprehensive evaluation of CPE's cytotoxic and anti-proliferative effects in the human TNBC model. Nevertheless, these findings reinforce the potential of CPE to enhance and maintain the anti-proliferative effects of standard chemotherapeutic agents, such as Dox. Analysis of several parameters involved in cancer eradication is needed to reveal the mechanism of CPE cytotoxicity in cancer cells, especially TNBC.

Migration is when cancer cells move to surrounding tissue near the primary cancer site. Migration is an important step that is strongly correlated with metastasis activity. Triple-negative breast cancer (TNBC) is known for its strong metastatic activity (Vuger et al. 2020; Schrörs et al. 2020). To test the ability of CPE, Sin, and Dox to suppress TNBC migration, a scratch-

wound healing assay technique was performed on 4T1 cells. This study found that Dox achieved the highest %closure, almost the same as untreated-scratched-4T1 cells, after 24 or 48 hours of treatments. This result is in line with the previous experiment (Amalina et al. 2023). Therefore, it is suggested that a combination of Dox with other agents that can inhibit Dox-induced migration is needed.

Interestingly, CPE exhibited stronger migration inhibition on 4T1 cells after 24 and 48 hours of treatment. Research on various pure citrus flavonoids in cancer cells has shown their ability to attenuate migration (Shi et al. 2013; Nurhayati et al. 2020; Zhu et al. 2024). A molecular approach was also conducted in this study to understand how CPE restricts 4T1 cell migration. Matrix metalloproteinase 9 (MMP-9), a crucial enzyme that facilitates cancer cell migration and invasion by degrading the extracellular matrix (ECM), was analysed for its activity using the gelatine zymography technique (Tafrihani et al. 2023). This study revealed that the activity of MMP-9 in CPE at 250 µg mL^{-1} and its combination with Dox at 1 µM was significantly suppressed compared to the untreated group. These findings suggest the prospect of CPE as a co-chemotherapeutic agent of Dox in suppressing breast cancer cell migration, particularly in TNBC.

CONCLUSION

CPE and Sin, which are CPE constituents, are known to have low and moderate cytotoxicity towards 4T1. CPE has been shown to increase the sensitivity of 4T1, a TNBC model, to Dox in a synergistic manner. CPE alone and in combination with DOX show an irreversible effect on 4T1 cells by inhibiting the formation of new colonies after 10 days without treatment. CPE and Sin can strongly inhibit the migration of 4T1 cells at 24 and 48 hours compared to the Dox-treated and untreated cells. CPE and its combination with Dox were shown to reduce the MMP-9 activity, which potentially reduces the cells' ability to degrade the extracellular matrix (ECM), leading to cell invasion inhibition. This study emphasises the CPE as a prospective-co-chemotherapy agent for several standard chemotherapeutic drugs.

AUTHORS CONTRIBUTION

SKZ, along with APP, collected and analysed the in vitro data. DRR, FAR, and ANA conducted the molecular assays. SKZ wrote the manuscript under the supervision of EM and RAS. RAS and EM contributed to the experimental design and provided supervision for the entire assay and manuscript writing.

ACKNOWLEDGMENTS

We are grateful for the support of the Rekognisi Tugas Akhir (RTA) program (under the official assignment letter number of 5286/UN1.P1/PT.01.03/2024) held by Universitas Gadjah Mada for this work.

CONFLICT OF INTEREST

All contributors to this work stated that there is no conflict of interest.

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