Chromolaena odorata L. Leaf Extract Elevates Cytotoxicity of Doxorubicin on 4T1 Breast Cancer Cells

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Abstract

Chemotherapeutic agents for breast cancer such as doxorubicin can attack normal cells as the side effects. *Chromolaena odorata* L. and its chemical content, sinensetin, have potential anticancer and antioxidant properties. The objective of this research is to examine the anticancer properties of *C. odorata* leaves extract and sinensetin on 4T1 triple negative breast cancer (TNBC) cells combined with doxorubicin. The MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5 diphenyltetrazolium bromide) assay on 4T1 cells was used to determine the IC_{50} and the Combination Index (CI) of the two agents in combination. Washing out the treatment and determining the cells viability after a few days was done to evaluate the persistence of the effects to cancer cells. *Chromolaena odorata* extract (COE) obtained was proven to contain sinensetin which gave a positive signal on the chromatogram. COE and sinensetin were moderately cytotoxic to 4T1 cells with IC_{50} value of 53 µg/mL and 58 µM (21.6 µg/mL), respectively. Both compounds were synergist (CI<0.7) to strong synergist (CI<0.3) when combined with doxorubicin (IC_{50} 90 nM = 0.05 µg/mL). COE and sinensetin exhibited moderate and not cytotoxic against Vero cells with IC_{50} values of 60 µg/mL and 243 µM (90.43 µg/mL), respectively. Both COE and sinensetin showed selectivity index values of >1 (1.13 and 4.19, respectively). Moreover, the cytotoxic effects of COE on 4T1 cells was persisted until 48 h after removing COE from the medium, indicating the tumor-suppression potency of COE. Our findings strengthen the scientific basis of *C. odorata* leaves extract to be developed as a co-chemotherapeutics agent for doxorubicin on TNBC.

Keywords: Chromolaena odorata *L., breast cancer cells, doxorubicin, co-chemotherapy, kidney cells.*

Submitted: November 13, 2023
Revised: December 19, 2023
Accepted: December 21, 2023
Published online: January 16, 2024

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Indonesian Journal of Cancer Chemoprevention, October 2023
ISSN: 2088–0197
e-ISSN: 2355-8989

INTRODUCTION

Doxorubicin is commonly used as a chemotherapeutic agent for cancer treatment which can kill and inhibit the proliferation of cancer cells. However, doxorubicin is not selective to the cancer cells and causes toxicity to the normal cells such as inducing senescence in normal cells (Bientinesi, *et al*., 2022). Moreover, many side effects of doxorubicin occur to patients including cardiac toxicity (Rawat, *et al*., 2021), pain, nausea, vomiting, hair loss (Febriansah & Lakshita, 2021), and also nephrotoxicity (Haryanti, *et al*., 2022). To reduce those side effects, companion agents (co-chemotherapy) are needed. The co-chemotherapeutic agent should have the anticancer activity and reduce the side effects when combined with the chemotherapeutic agent (Mulyati, *et al*., 2017). Agents from natural ingredients could potentially be the candidate for co-chemotherapy.

The development of co-chemotherapy for doxorubicin has shown promising results from natural ingredients such as plants. We noted that rice bran extract exhibits synergistic effects with doxorubicin to suppress the growth of 4T1 triple negative breast cancer (TNBC) cells. The synergistic effects also correlated with the induced cell senescence but did not increase intracellular reactive oxygen species (ROS) accumulation. Interestingly, this cytotoxic effect does not occur in normal fibroblast cells (Zulfin, *et al*., 2021). This phenomenon is also found in the combination of galangal extract and doxorubicin against 4T1 cancer cells which show synergism in cancer cells with increasing ROS intracellular level but downregulate the ROS intracellular level and senescence in normal fibroblast cells (Ahlina, *et al.*, 2020). These studies show the potential properties as co-chemotherapeutic agents of some natural ingredients, but further researches are important to find agents which have more potential, easy to be collected, and safe for normal cells.

In this study we investigate *Chromolaena odorata* L., commonly known as devil weed or "kirinyuh" in Indonesia, a type of wild bush plant and invasive for agricultural and forest environment (Okoro, *et al*., 2019). This plant is spread all over the world in almost all types of habitats (Aziz, *et al*., 2020). This plant contains acacetin, chalcones, eupatilin, luteolin, scutellareintetramethyl ether, and sinensetin that have been known to exhibit antibacterial activity, anti-fungi, and antioxidant properties (Atindehou, *et al*., 2013; Sirinthipaporn & Jiraungkoorskul, 2017; Zahara, 2019; Tahir, *et al*., 2021). *C. odorata* is easy to find and has potential source of compounds to be used as a co-chemotherapeutic agent. In addition, *C. odorata* also has anticancer properties with cytotoxicity in cancer cells such as cell cycle arrest, autophagy, and apoptosis (Yusuf, *et al*., 2021; Olawale, *et al*., 2022).

Sinensetin elicits anticancer effects on gallbladder adenocarcinoma cells by inhibiting proliferation, migration, and invasion, also inducing apoptosis (Huang, *et al*., 2020). This compound is also able to inhibit cell proliferation by inducing apoptosis and autophagy in leukemia cells (Tan, *et al*., 2019). In addition, this compound has low cytotoxicity with IC_{50} of 0.4-88.9 μM and high selectivity on normal cells (Jie, *et al*., 2021). Those characteristics of sinensetin show the co-chemotherapy potential of *C. odorata* extract and sinensetin as the possibly main active compound of *C. odorata*. This research aims to further validate the co-chemotherapy potency of *C. odorata*, that has not been established before, by evaluating their cytotoxic activity and their synergism with doxorubicin against 4T1 TNBC cells, as well as their selectivity on normal cells.

METHODS

Ethical Issues

All of these experiments were approved by Ethical Clearance from the Medical and Health

Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (UGM) (reference number KE/FK/1231/EC/2023).

Extraction and Profiling of *Chromolaena odorata* Leaves

The collected *C. odorata* leaves from Kalasan, Yogyakarta was determined by the Department of Pharmaceutical Biology, Faculty of Pharmacy Universitas Gadjah Mada (UGM) (reference number 51.14.8/UN1/FFA.2/BF/PT/2023). Approximately 3.2 kg of leaves were washed with water, rinsed, and dried using an oven for 40°C for 24 h. The dried leaves were ground by grinder and sifted by 60 mesh sieves to obtain 629 gram of leaves powder. The extraction of *C. odorata* leaves powder was done by macerating them in 96% ethanol for 24 h. The ethanol was then evaporated in the fume hood until a thick extract with a stabile weigh obtained. The extract is mentioned as *Chromolaena odorata* extract (COE) from here after. Profiling of the extract was done by thin layer chromatography (TLC) using Silica Gel plate F254 for the stationary phase and mixture of toluene and ethyl acetate (5:7) for the mobile phase as modified from Kartini, *et al*. (2023). The profiling then visualized under 366 nm UV. Sinensetin (SML1787, Sigma-Aldrich) with a purity of ≥98% was employed as the standard.

MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5 diphenyltetrazolium bromide) Assay

Triple negative breast cancer (TNBC) cells 4T1 (ATCC CRL-2539) and normal kidney cells Vero (ATCC CCL-81) were collection of Cancer Chemoprevention Research Center (CCRC) Faculty of Pharmacy UGM. The cells were cultured in the complete medium that consists of Dulbecco's Modified Eagle Medium (DMEM) with the addition of 10% fetal bovine serum (FBS) and antibiotics. The cells were planted in a 96-well plate with 5×10^3 cells for each well and treated by serial concentration of COE (1, 2.5, 5, 10, 25, 50, and 100 µg/mL), sinensetin (SML1787, Sigma-Aldrich) with a range of 1-100 µM (that was equal to 0.37, 0.93, 1.85, 3.72, 9.31, 18.62, and 37.24 µg/mL), or doxorubicin (046-21523, Fujifilm Wako) with a range of 0.1-10 µM (equal to 0.06, 0.14, 0.29, 0.58, 1.44, 2.89, and 5.8 µg/mL) for 24 h. The cell viability for cytotoxic activity of single and combination agents was measured by MTT assay using MTT reagent. Ten percent of sodium dodecyl sulfate in 0.01 N HCl as the stopper reagent was added to dissolve the formed formazan. The absorbance was measured at 595 nm using a microplate reader (Hanifa, *et al*., 2022). The concentration that inhibits 50% of cell viability (IC_{50}) value of the agents was obtained and used for the combination assay (⅛, ¼, and ½ of IC_{50} for COE or sinensetin and doxorubicin).

The Combination Index (CI) was determined (Ikawati, *et al*., 2018) by using the following equation from Reynolds & Maurer (2005), whereas D is the concentration of the compound in the combination assay and Dx is the calculated concentration of the single compound derived from the single linear regression equation to obtain the particular cell viability as resulted in the combination assay.

$$CI=D1/Dx1+D2/Dx2$$

The IC_{50} values were also used for determining the Selectivity Index (SI) by calculating the ratio between IC_{50} value in cancer cells and normal cells (Indrayanto, *et al*., 2020). The synergistic of the combination agents were interpreted by CI score from the cell viability (Musyayyadah, *et al*., 2021).

MTT assay was also used for evaluating the persistence of the samples by planting 4T1 cells in a 96-well plate with 4×10^3 cells for each well. After 24 h incubation, the cells were treated with serial concentration of COE 20-240 µg/mL and incubated for 24 h. Phosphate buffered saline (PBS) was used to wash the remains of COE-containing medium (wash-out) and the medium was replaced by the media only. The cell viability was determined

Indonesian Journal of Cancer Chemoprevention, October 2023
ISSN: 2088–0197
e-ISSN: 2355-8989

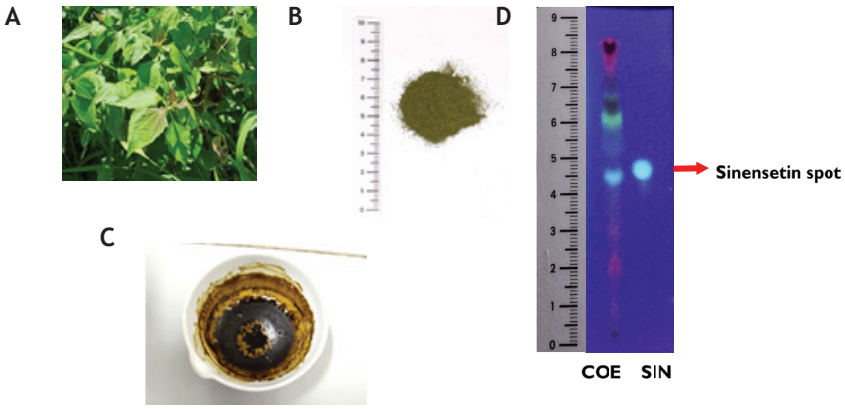


Figure 1. Extract and the TLC analysis. (A) *Chromolaena odorata* L. plants, (B) *C. odorata* powder, (C) *C. odorata* extract (COE) with yield 7.70% obtained by 96% ethanol maceration, (D) TLC profiling of COE and sinensetin (SIN) as the standard. The red arrow indicates sinensetin spot.

by MTT assay for each day until 48 h after the wash-out (Larasati, *et al.*, 2018). The significance of the cell viability percentage between COE-treated cells and untreated cells was analyzed statistically using Student's t-test (SPSS).

RESULTS

Extraction and Profiling of *Chromolaena odorata* Extract (COE)

The extract with a yield of 7.7% was obtained in a semisolid form (thick extract) (Figure 1C) and then was diluted in ethanol for chromatography profiling. A spot in COE profiling was visible beside the sinensetin spot as the standard with similar color under the 366 nm UV light (Figure 1D). Thus, it is feasible that COE contained sinensetin as one of the ingredients.

Cytotoxicity of *Chromolaena odorata* Extract (COE) and Sinensetin

COE showed growth suppressing effects against 4T1 cells in a dose dependent manner with the IC_{50} value of 53 µg/mL (Figure 2A), whereas sinensetin exhibited cytotoxic effects with the IC_{50} of 58 µM (21.6 µg/mL) (Figure 2B). Doxorubicin

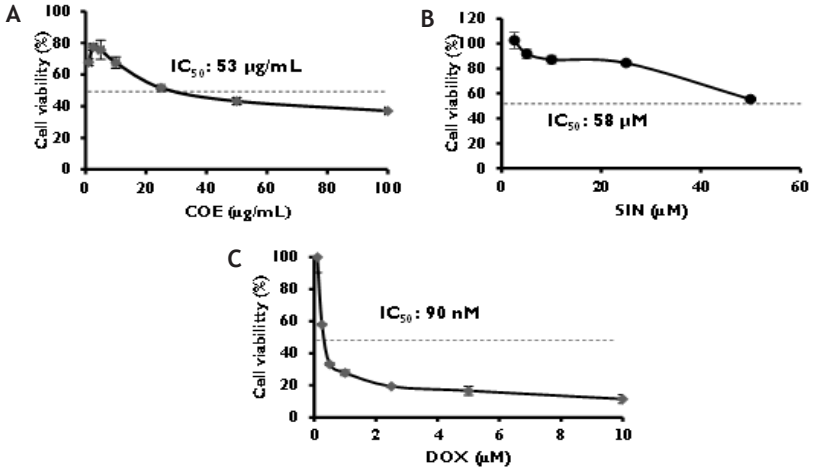


Figure 2. Cytotoxic activities of COE (A), sinensetin (B), and doxorubicin (C) on 4T1 cells. The cytotoxic effect of the compounds was determined using MTT assay after a 24-h treatment. The data are shown as average ±standard error (SE) (*n*=3). The dashed line marks the 50% cell viability. SIN: sinensetin. DOX: doxorubicin.

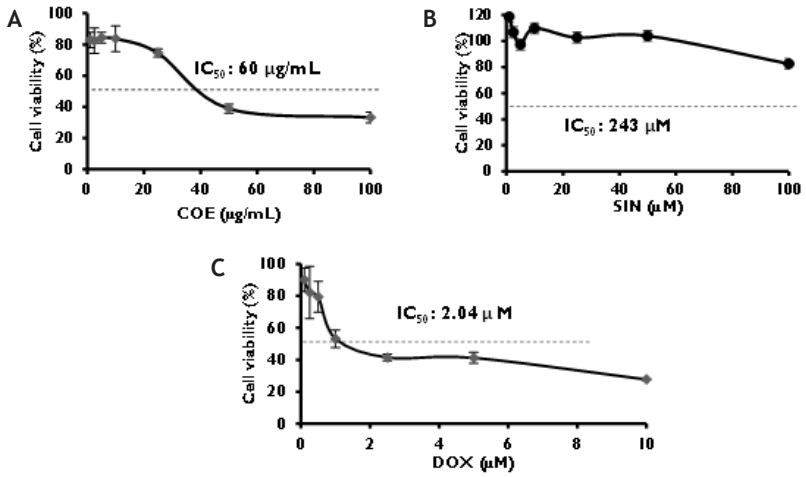


Figure 3. Cytotoxic activities of COE (A), sinensetin (B), and doxorubicin (C) on Vero cells. The cytotoxic effect of the agents after a 24-h treatment was determined using MTT assay. The data are shown as average ±SE (*n*=3). The dashed line marks the 50% cell viability. SIN: sinensetin. DOX: doxorubicin.

Table 1. Cytotoxic activity and Selectivity Index (SI) of the samples towards 4T1 and Vero cells.

Sample	IC_{50}		SI
	4T1	Vero	
COE	53 µg/mL	60 µg/mL	1.13
Sinensetin	58 µM (21.6 µg/mL)	243 µM (90.49 µg/mL)	4.19
Doxorubicin	0.09 µM (0.05 µg/mL)	2.04 µM (1.18 µg/mL)	22.7

gave the strongest cytotoxicity with the IC_{50} value of 90 nM (0.05 µg/mL) (Figure 2C). Both COE and sinensetin were categorized as moderately cytotoxicity based on the classification from the World Health Organization (2017) in which IC_{50} value between 2-89 µg/mL is categorized as moderately cytotoxic. These data proved that COE and sinensetin has cytotoxic potential towards 4T1 breast cancer cells.

On the other hand, the cytotoxic activity of the compounds toward non-cancerous cells Vero was also evaluated to determine the SI. The results showed a higher value of IC_{50} for all the three tested compounds (Figure 3). Sinensetin showed the highest IC_{50} value on Vero cells with the extrapolated IC_{50} of 243 µM (90.49 µg/mL), followed by COE with the IC_{50} of 60 µg/mL. The IC_{50} of normal cells was then calculated with the IC_{50} of cancer cells to obtain SI, which is the ratio of IC_{50} values of cancer cells and non-cancer cells. A compound is considered to be selective to cancer cells if the SI value is equal to or higher than 10 (Pena-Moran, *et al*., 2016). In these results, COE and sinensetin were not considered as selective to the cancer cells (Table 1).

The persistence of COE's cytotoxic activity was evaluated through wash-out experiment (Larasati, *et al*., 2018) and the cell viability was again determined using MTT assay every 24 h. The viability of 4T1 cells decreased significantly

Indonesian Journal of Cancer Chemoprevention, October 2023
ISSN: 2088–0197
e-ISSN: 2355-8989

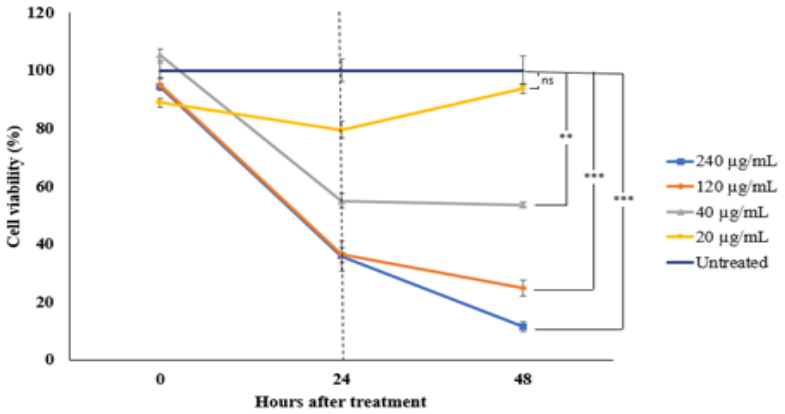


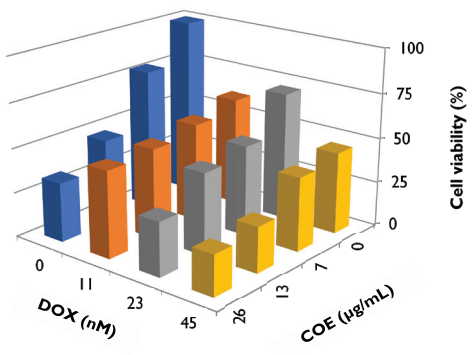
Figure 4. The persistence of COE's cytotoxic effect on 4T1 cells after 48 h. The cells were plated in a 96-well plate and were incubated with a serial concentration of COE. After 24 h, the media containing extract were replaced by media only after PBS washing and the cell viability was determined by MTT assay at the indicated time points. ns: not significant; ** p<0.01; *** p<0.001.

until 48 h after washout start from 40 µg/mL group (Figure 4). This finding indicates that COE has a good potential in suppressing tumor growth.

Combination Potencies of *Chromolaena odorata* Extract (COE) and Sinensetin with Doxorubicin

After determining the cytotoxic activity for each agent, we then determined the cytotoxic activity for combination of COE plus doxorubicin and sinensetin plus doxorubicin. These experiments were executed to obtain the optimal concentration for next experiments by determining the CI for those combinations. The cytotoxic activity of COE and doxorubicin combination showed synergistic effects with the lowest and the highest CI score of 0.14 and 0.36 (Figure 5). The combination potency is interpreted as synergist for CI of 0.3-0.7 while strong synergist for CI of 0.1-0.3 (Reynolds & Maurer, 2005). The cytotoxic activity of sinensetin and doxorubicin combination showed synergistic effects with the CI score ranging from 0.00 to 0.47 (Figure 6). Thus, it can be concluded that sinensetin, similar to COE, had a very good synergism to increase the cytotoxicity of doxorubicin on 4T1 breast cancer cells.

A

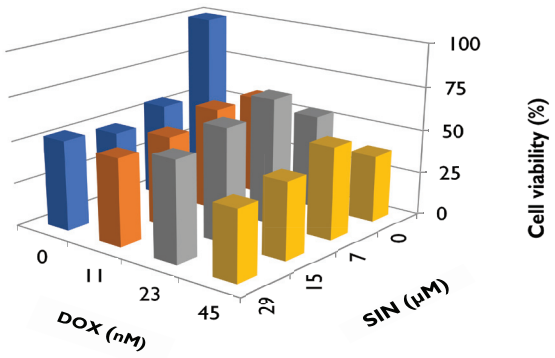


B

COE (µg/mL)	Doxorubicin (nM)		
	11	23	45
7	0.16	0.19	0.18
13	0.29	0.22	0.14
26	0.36	0.23	0.22

Figure 5. The cytotoxicity of COE and doxorubicin combination on 4T1 cells (A) and the Combination Index (B). The cells were grown in a 96-well plate (5×10^3 cells/well). The treatment was performed with sub-dose IC_{50} (⅛, ¼, and ½) of doxorubicin (DOX) (11, 23, 45 nM) and COE (7, 13, 26 µg/mL) for 24 h. The cell viability was then counted by MTT assay.

A



B

Sinensetin (µM)	Doxorubicin (nM)		
	11	23	45
7	0.14	0.00	0.22
15	0.47	0.37	0.26
29	0.44	0.46	0.44

Figure 6. The cytotoxicity of sinensetin and doxorubicin combination on 4T1 cells (A) and the Combination Index (B). The cells were grown in a 96-well plate (5×10^3 cells/well). The treatment was performed with sub-dose IC_{50} (⅛, ¼, and ½) of doxorubicin (11, 23, 45 nM) and sinensetin (7, 15, 29 µM) for 24 h. The cell viability was then counted by MTT assay. DOX: doxorubicin. SIN: sinensetin.

DISCUSSION

Chromolaena odorata are known as weed plants and have been used as traditional medicine (Sirinthipaporn & Jiraungkoorskul, 2017). This plant contains a variety of compounds such as flavonoids, and sinensetin is concluded as one of the flavonoids in the leaves extract (Atindehou, *et al.*, 2013). This potential plant is proposed to challenge the lesser benefit of doxorubicin as co-chemotherapeutic agent against metastatic breast cancer cell, 4T1. This study gives insight for developing alternative medication for breast cancer that is still dependent on doxorubicin and resulting in a lower dose of doxorubicin to reduce its adverse effects. In addition, we also include sinensetin as the main known compound of *C. odorata* to provide the co-treatment activity as a comparison.

Our results show that COE and sinensetin are considered as moderately cytotoxic on 4T1, whereas in Vero cells they are moderately or not cytotoxic. However, with SI values >1, the concentration of both COE and sinensetin to achieve therapeutic effect is lower than the concentration that causing toxic effects or undesired effects on normal cells, as previously stated (Sholikhah, *et al.*, 2018), thus they are prospective as chemopreventive agents. However, we should consider that the IC_{50} value of sinensetin as a single compound is quite high, meaning that the potential cytotoxic activity of sinensetin is categorized as low if we compared to doxorubicin that performed more than 100 times its cytotoxic activity. As an extract, CEO with the IC_{50} below 100 µg/mL is prospective to explore further to find out the active compound regardless of sinensetin. Compared to galangal extract and rice bran extract (Ahlina, *et al.*, 2020) for example, the COE has stronger cytotoxic activity towards 4T1 cancer cells. The potential anticancer property of COE is also supported by the persistence cytotoxic activity in the wash out experiment. In this concern, some active compounds of CEO may contribute to this activity by irreversibly binding to some essential proteins in cancer development that are interesting to be investigated further.

COE and sinensetin have low cytotoxic activities but they show synergism with doxorubicin to suppress the growth of 4T1 cells. We found that COE and doxorubicin are interpreted as strong synergism, while sinensetin and doxorubicin are interpreted as synergism, meaning that other compounds in COE probably play role in its cytotoxicity and have better effect than sinensetin. This concluded that COE has more potential than sinensentin to be combined with doxorubicin. Compared to the cytotoxic combination with galangal extract (Ahlina, *et al.*, 2020), the combination from COE have lower concentration in

combination treatment with doxorubicin, indicating that COE have more effective efficacy to be co-chemotherapy with doxorubicin. We also found that COE combination with doxorubicin have better efficacy than rice bran extract and soursop leaves extract combined with doxorubicin (Zulfin, *et al*., 2021; Salsabila, *et al*., 2021). However, further research is needed to determine the best concentration combination of the two agents to find the most effective way to downregulate the cancer growth but less toxic to the normal cells. Moreover, the study about the migratory inhibition of TNBC cells by COE and sinensetin might be needed for the development of co-chemotherapeutic agents against highly metastatic breast cancer cells, for example by using the scratch wound healing assay, gelatin zymography, and morphological lamellipodia formation (Amalina, *et al*. 2023). Since 4T1 is a murine-derived cell line, a further research by using human TNBC cells, *i..e* MDA-MB-231 (Novitasari, *et al*., 2022) in the development research for breast cancer co-chemotherapeutic agents is also necessary.

CONCLUSION

We confirmed that COE contains sinensetin. Both COE and sinensetin are moderately cytotoxic to 4T1 breast cancer cells. COE and sinensetin synergistically improve the cytotoxicity of doxorubicin in 4T1 cells. COE and doxorubicin have stronger synergisms than sinensetin and doxorubicin combination. Moreover, COE maintains the cytotoxicity at least up to 48 h after its removal from the cells. Taken together, COE is promising to be further examined as a co-chemotherapy agent for doxorubicin.

ACKNOWLEDGEMENTS

This research was funded by the Directorate of Research, Technology, and Community Services, the Directorate General of Higher Education, Research, and Technology, the Indonesian Ministry of Education, Culture, Research, and Technology (no. 122/E5/PG.02.00.PL/2023) via the master thesis research scheme [Penelitian Tesis Magister] (no. 3203/UN1/DITLIT/Dit-Lit/PT.01.03/2023). We thank all members of CCRC Faculty of Pharmacy UGM for their support in this study.

AUTHOR CONTRIBUTIONS

Conceptualization, E.M. and M.I.; methodology, E.M. and M.I.; data curation, A.P.P. and D.R.R.; writing—original draft preparation, A.P.P and M.I.; writing—review and editing, A.P.P., E.M., and M.I.; supervision, E.M. and M.I.; funding acquisition, M.I. All authors have read and agreed to the published version of the manuscript.

FUNDING

The Directorate of Research, Technology, and Community Services, the Directorate General of Higher Education, Research, and Technology, the Indonesian Ministry of Education, Culture, Research, and Technology (no. 122/E5/PG.02.00.PL/2023) via the master thesis research scheme [Penelitian Tesis Magister] (no. 3203/UN1/DITLIT/Dit-Lit/PT.01.03/2023).

CONFLICTS OF INTEREST

The authors declare no conflict of interest. The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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