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Duku peel ethyl acetate extract as an adjunctive treatment of doxorubicin on triple negative breast cancer 4T1 cells

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ABSTRACT Triple-negative breast cancer (TNBC) faces significant treatment challenges due to its resistance to chemotherapy and high-rate metastatic occurrence. Adjunctive treatment is a promising approach to improve chemotherapy effectiveness while reducing toxicity on normal cells. Natural compounds of *Lansium domesticum* (duku) exhibit potential as adjunctive cancer treatments. This study aims to investigate the potential of duku peel extract (DPE) to be developed as an adjunctive agent. The cytotoxic activity of DPE against 4T1 cells was conducted through MTT assay, both in single and combination treatments. The anti-migratory effect of DPE was examined by scratch wound healing assay. The molecular mechanism of DPE was confirmed using virtual screening via bioinformatics approaches, including protein target prediction and molecular docking. The results show that DPE has cytotoxic activity with an IC_{50} value of 47 µg/mL against the 4T1 cell line, in a 24-hour treatment period. Interestingly, DPE not only had a good synergistic effect (mean CI value, i.e. 0.34), but also showed significant inhibition of cell migration with doxorubicin (Dox). Additionally, virtual bioinformatics screening approaches suggest the potential mechanism of DPE compound action by targeting CDC25B and TOP2A. Overall, DPE holds promise as an adjunctive treatment of Dox against 4T1 TNBC cells.

KEYWORDS Adjunctive treatment; Bioinformatics; *Lansium domesticum*; Migration; TNBC

1. Introduction

Breast cancer is the most common cancer in women worldwide (Manjunath and Choudhary 2021), with a mortality rate of 6.8% (Sung et al. 2021). Triple-negative breast cancer (TNBC) is one of the subtypes of breast cancer, accounting for 15–25% of all types of breast cancer (Almansour 2022). The characteristics of TNBC are low expression of human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR), making hormonal and anti-HER2 therapy unable to be used to treat in this subtype of cancer (Won and Spruck 2020). Apart from being difficult to treat, this subtype of cancer also shows a worse prognosis than other subtypes (Won and Spruck 2020). Due to the low expression of these protein receptors, the treatment for TNBC therapy is non-specific chemotherapy, which induces several side effects (Mollah and Varamini 2021). Another challenge in treating TNBC is because of its high-rate metastatic occurrence (Singh and Yadav 2021). Doxorubicin (Dox) is one of the effective chemotherapeutic agents including TNBC by causing DNA damage, leading to the death of cells. However, Dox has limitations because of its long side-effect such as cardiotoxicity (van der Zanden et al. 2021).

Adjunctive treatment or co-chemotherapy is an approach to enhance the effectiveness of chemotherapy while reducing its toxicity on normal cells (Meiyanto et al. 2012). Natural resources such as plant give promising effect to enhance the efficacy of chemotherapy. Various plants resources such as *Caesalpinia sappan* L., *Alpinia galanga* L., *Piper nigrum* L., and rice bran extracts has been investigated as adjunctive treatment in several cancer cell lines and have been reported to poses a promising anticancer drug (Alif et al. 2021; Nugraheni et al. 2021; Zulfin et al. 2021; Haryanti et al. 2022). In this study, we propose *Lansium domesticum* (locally known as "duku" in Indonesia) to be developed as an adjunctive treatment agent for TNBC.

Duku, commonly grown in Southeast Asia, is a plant from the Meliaceae family (Mayanti et al. 2023). Its peel is known to have various phytochemicals, such as lansic acid, lansioside B, and lansiolic acid, which are triter-

penoid compounds (Abdallah et al. 2022). Three isolated compounds from duku peel, i.e. 3-hydroxy-8,14-secogammacera-7,14-dien-21-one, lamesticumin A, and kokosanolide E, were shown to have moderate cytotoxic activity against MCF-7 and T47D cell lines (Fadhilah et al. 2021; Labibah et al. 2021; Mayanti et al. 2023). Those compounds suggest that duku peel may possess anticancer activity. However, the cytotoxic effect of duku peel extract (DPE) and its combination with Dox on TNBC cell line has not been performed yet. Therefore, we focused on the cytotoxic and anti-migration activity of DPE, using ethyl acetate solvent, and its combination with Dox against the TNBC cancer model, 4T1.

This study aims to investigate the potential of DPE to be used as an adjunctive agent with Dox against TNBC. We employ 4T1 cells as representatives of TNBC. The cytotoxicity of DPE using MTT assay was performed to determine the IC_{50} value and its anti-migratory effect through scratch wound healing assay. Dox was employed as a chemotherapy agent for *in vitro* studies. The synergistic effect of DPE in combination with Dox was determined by calculating the combination index (CI) value. Additionally, we analyzed the potential mechanism of DPE through bioinformatics approaches, including protein target prediction and molecular docking.

2. Materials and Methods

2.1. Sample preparation and triterpenoid identification

The fruits of duku (*Lansium domesticum*) was obtained from Small and Medium Enterprises in Sleman, Daerah Istimewa Yogyakarta, Indonesia. The sample was authenticated by a specialist at the Faculty of Pharmacy, Universitas Gadjah Mada. The peels were dried in a dehydrator at around 40 °C, then ground and sieved to a 40-mesh size to obtain duku peel powder. A total of 0.92 g of the powder was weighed and macerated with ethyl acetate *p.a.* (1:10 ratio) assisted with a rotary shaker. overnight. The resulting mixture was separated from the residual powder, and the duku peel extract (DPE) was evaporated to obtain dried extract, yielding 0.177 g of dried extract, corresponding to a 19% w/w yield value. The DPE was prepared for an *in vitro* experiment.

2.2. Cell culture

The 4T1 cells were obtained from Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy UGM. The medium used for cell culture was high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, New York, USA). The fetal bovine serum (FBS) 10% v/v and penicillin-streptomycin (Gibco) were added to the medium. The incubation condition was at 37 ºC with 5% CO_2.

2.3. Cytotoxic activity on 4T1 cells

The cytotoxicity of DPE on 4T1 cells was performed using MTT assay. The 4T1 cells (5×10^3/well) were seeded in a 96-well plate, then incubated overnight. The cells were treated using a series concentration of 1-500 µg/mL DPE and 0.01–10 µM Dox for 24 h. In the cytotoxic combination setting, the concentration used was ½, ¼, and ⅛ of the IC_{50} of both DPE alone and in combination with Dox, and incubated for 24 h. The treated cells were washed using phosphate-buffered saline (PBS, Sigma) and then added with MTT solution for 3–4 h until the formazan crystal formed. The stopper solution (SDS 10% in HCl 0.01 M) was added after the crystal formed and incubated overnight in the dark room (Zulfin et al. 2021). The absorbance was measured using a microplate reader (Bio-Rad) at 595 nm. The percentage of viability cells was analyzed from the absorbance to measure the IC_{50} value by using linear regression (95% confidence), followed up by the CI calculation using Chuo-Talalay method as follows:

$$CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} \quad (1)$$

$(D)_1$ and $(D)_2$ represents concentration of sample 1 and sample 2, respectively, while Dx represent concentration of a single drug that achieves an effect comparable to that produced by a combination treatment (Chou 2010).

2.4. Scratch wound healing assay

The anti-migration activities of DPE and Dox both single and combination on 4T1 cell, were assessed using scratch wound-healing assay, following the protocol established previously (Wulandari et al. 2021). The cells (7.2×10^4/well) were seeded in a 24-well plate, then incubated overnight. A day after, the medium was changed into starvation medium (1 µg/mL mitomycin, 0.5% FBS) and incubated for 18 h. The cells were washed with PBS (Sigma Aldrich). The cells were scratched using a sterile yellow pipette tip. Subsequently they were treated with both single and combination of DPE and Dox (at concentration ¼, and ⅛ IC_{50}), and simvastatin (Sim) was used as a positive control due to its proven ability to inhibit cancer cell migration (Tripathi et al. 2023). The wound closure was captured using an inverted microscope at a 24-h and 48-h interval and calculated % wound closure using ImageJ software (version 1.54).

2.5. Protein target prediction analysis

The protein target prediction analysis was retrieved by collecting the top 250 over-expressed genes in TNBC from UALCAN (https://ualcan.path.uab.edu/) and identifying potential protein targets based on molecule structure from SwissTargetPrediction (http://www.swisstargetprediction.ch/) (Daina et al. 2019; Chandrashekar et al. 2022). The potential compounds of DPE were retrieved from several literature studies that conduct extraction and isolation of DPE followed by cytotoxic testing against breast cancer cells (Fadhilah et al. 2021; Labibah et al. 2021; Mayanti et al. 2023). The molecule structure of potential DPE compounds was redrawn in SwissTargetPrediction, simultaneously obtaining their SMILES codes. The data list was compared to analyze the correlation using InteractiVenn

(https://www.interactivenn.net/). The protein identified across all data lists was subjected to further analysis to elucidate the protein-molecule interactions.

The protein-molecule interactions analysis was performed using the Molecular Operating Environment (MOE) version 2015.10 software licensed from the Faculty of Pharmacy UGM with a default setting. The DPE compounds were constructed using MOE, followed by energy minimization and conformational search. The crystal structure of protein targets was retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/). Molecular docking validation was accomplished by self-docking the crystal structure with its native ligand. The output scores of protein-molecule interactions were compared with the known protein inhibitor, which serves as the established native ligand in the crystal structure.

2.6. Statistical analysis

The statistical analysis was utilized using software Microsoft Excel 365 and Jamovi version 2.6.2. Linear regression was calculated by comparing *r* value with *r* table in Microsoft excel 365. The parameter for the scratch wound healing assay, percentage of wound closure, was analyzed for significant differences using ANOVA followed by Tukey Post-Hoc test in Jamovi 2.6.2. All the statistical analysis was conducted with a confidence interval of 95% ($p < 0.05$).

3. Results and Discussion

3.1. Cytotoxic activity of DPE and Dox

The cytotoxic activity of DPE was performed using MTT assay against the TNBC cell model, 4T1. One hundred percent (100%) cell viability indicates the number of viable cells in the intervention group have the same amount as the untreated group. The calculated IC_{50} values were 47 µg/mL and 0.35 µM for DPE and Dox, respectively (Figure 1). This data showed that both DPE and Dox have cytotoxic activity toward 4T1 cells. This IC_{50} value was utilized to determine the treatment concentration in further *in vitro* analysis, including combination cytotoxic assay with Dox and scratch wound healing assay.

3.2. DPE showed potential cytotoxic activity combined with doxorubicin

The synergistic impact of DPE in conjunction with Dox was assessed using the MTT assay. For each agent, we utilized serial concentrations below the IC_{50} value, specifically ⅛, ¼, and ½ of the IC_{50} value. The 4T1 cell viability decreased in concentration-dependent manner, with the combination treatment of the DPE and Dox exhibits more promising reduction compared to individual treatment (Figure 2a). This combination demonstrated a favorable synergistic effect, as indicated by a CI value of less than 1 (mean CI value = 0.34) (Figure 2b). These findings highlight that when combined with Dox, DPE effectively reduces 4T1 cell viability.

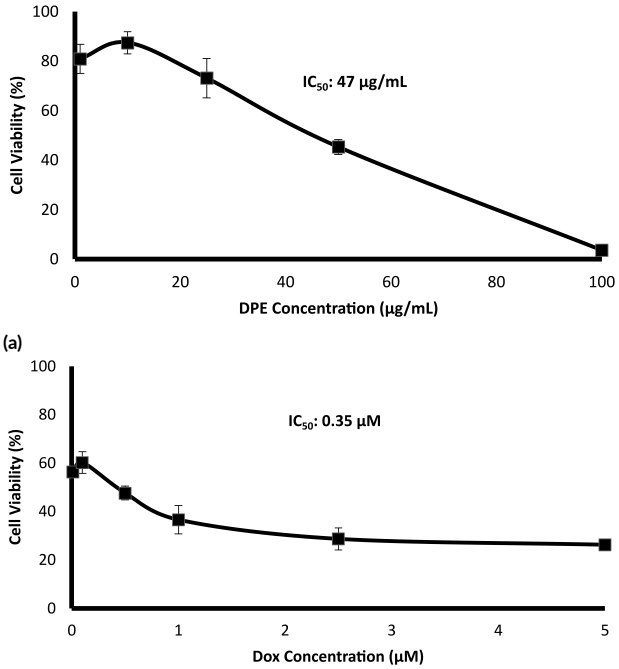
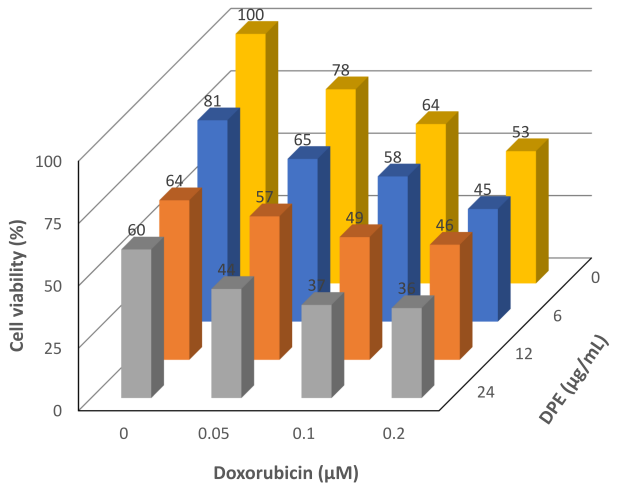


FIGURE 1 Cytotoxic effect of DPE (a) and Dox (b) on 4T1 cells. DPE and Dox had cytotoxic activity on 4T1 cells with IC_{50} of 47 µg/mL and 0.35 µM, respectively, obtained from linear regression calculations of concentrations vs cell viability ($p < 0.05$).



(a)

Doxorubicin (µM)	Duku Peel Extract (µg/mL)		
	6	12	24
0.05	0.18	0.26	0.48
0.1	0.05	0.44	0.43
0.2	0.29	0.43	0.46

(b)

FIGURE 2 The cytotoxic combination effect of DPE and Dox on 4T1 cells (a) and its combination index (b).

3.3. DPE inhibits the migration of 4T1 cells

The anti-migration activity of DPE was examined using scratch wound healing assay. The wound closure of 100% indicates that there is no remaining gap area in the time interval compared with gap area immediately after the scratch. A lower percentage of wound closure indicates a stronger anti-migratory activity exhibited by the sample. The anti-migration effect was observed using inverted microscope (Figure 3a). The concentration used was ⅛ and ¼ of IC_{50} to evaluate anti-migration property while minimize the cytotoxic effect. After 24 h, cells treated with Dox showed percentage of wound closure up to 75.4 ± 1.1% even 92.2 ± 0.2% after 48 h (Figure 3b and 3c). In the other side, cells treated with DPE both single and combination with Dox showed significant difference ($p < 0.05$) after 48 h treatment. Interestingly, DPE 24 µg/mL in combination with Dox showed significant difference ($p < 0.05$) compared to DPE alone.

3.4. Potential protein target of DPE

Research on isolated compounds from DPE has already been conducted. Among these compounds, several have been tested the potential against breast cancer cells. Those compounds were 3-hydroxy-8,14-secogammacera-7,14-dien-21-one (against T47D cells), lamesticumin A (against MCF-7 cells), and kokosanolide E (against T47D) (Fadhilah et al. 2021; Labibah et al. 2021; Mayanti et al. 2023). The potential protein target by those compounds was analyzed using SwissTargetPrediction (http://www.swisstargetprediction.ch/). The top 250 over-expressed genes in TNBC were collected from database UALCAN (https://ualcan.path.uab.edu/). The correlation of potential protein

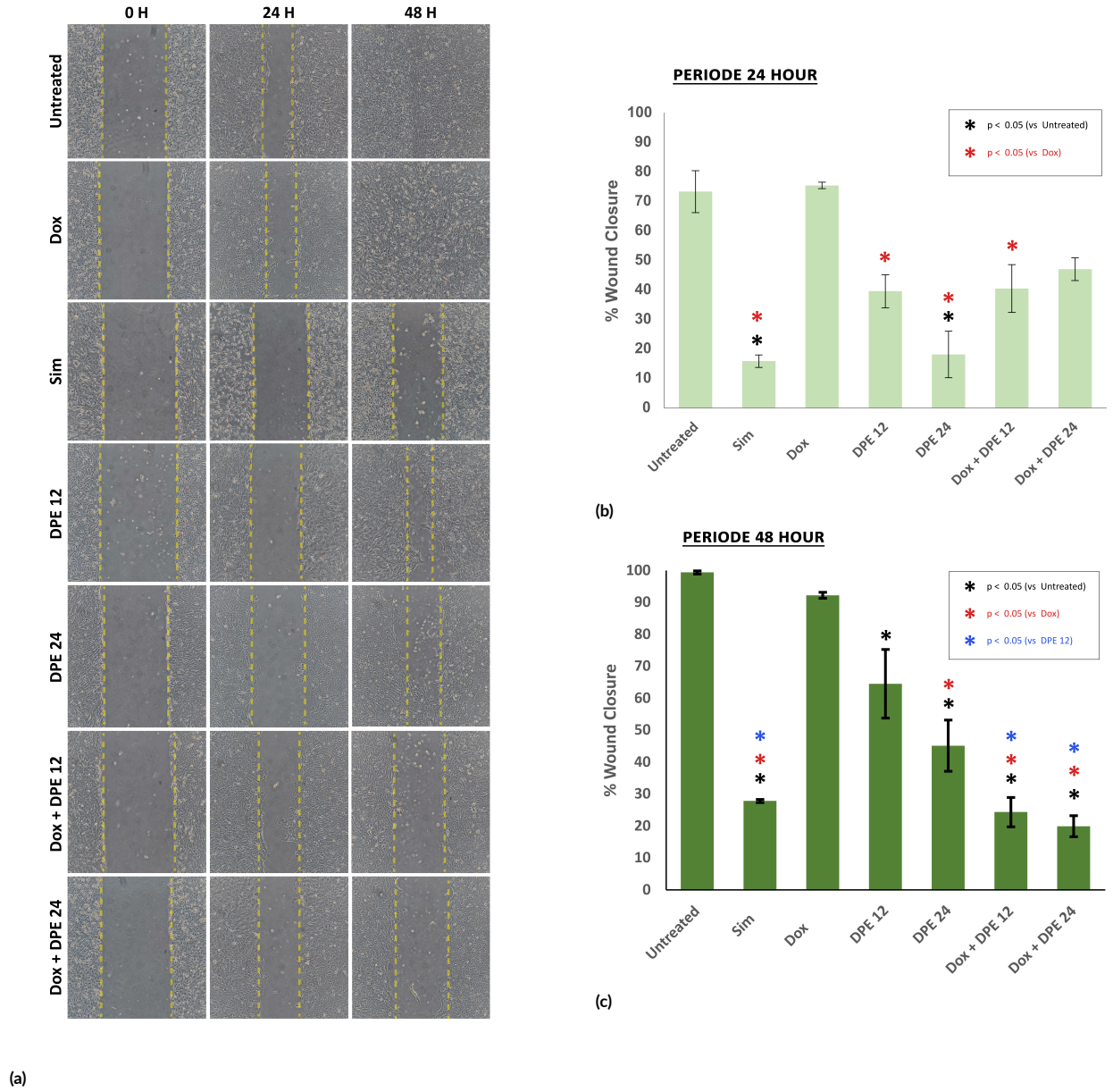


FIGURE 3 Anti-migration effect of DPE in 4T1 cells observed with an inverted microscope (a) at 24 h (b) and 48 h (c).

targets and over-expressed genes in TNBC was analyzed using Interactive Venn (https://www.interactivenn.net/). The potential protein targets analysis identified CDC25B and TOP2A (Figure 4). Furthermore, their interaction with DPE's compounds were analyzed using molecular docking.

The crystalized protein structure of CDC25B (PDB ID:4WH9) and TOP2A (PDB ID:5GWK) was retrieved from the database RCSB PDB (https://www.rcsb.org/). These crystalized proteins were chosen due to the existence of native ligand as inhibitor of the protein. The interaction between CDC25B with DPE compounds was stronger than with its native ligand, whereas interaction between TOP2A and DPE compounds was weaker than its native ligands, as indicated by the docking score (Figure 5a-c). The RMSD of the native ligand (Figure 5c) ≤ 2 Å, suggesting a good docking protocol (Verdonk et al. 2003). This suggests that DPE compounds potentially bind to CDC25B and inhibit its activity through a similar site and mechanism as its native ligand (Figure 5c).

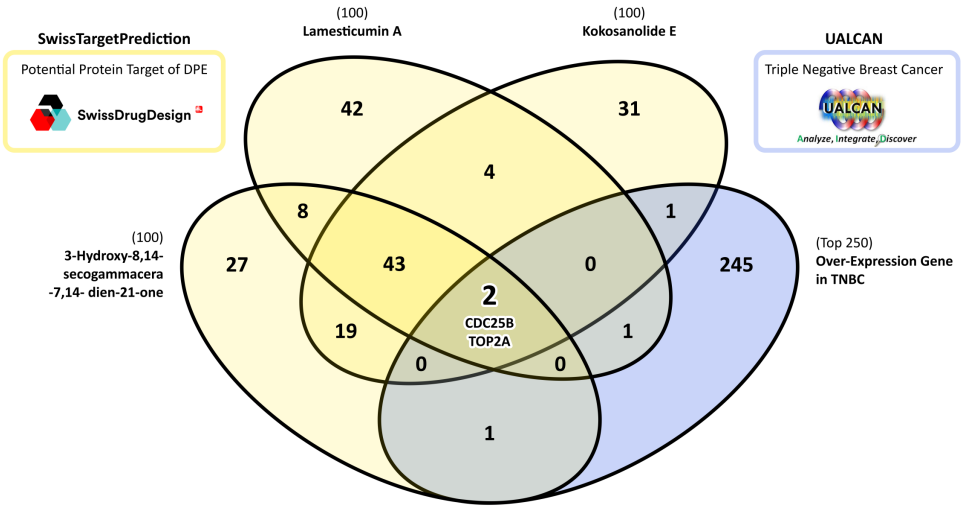
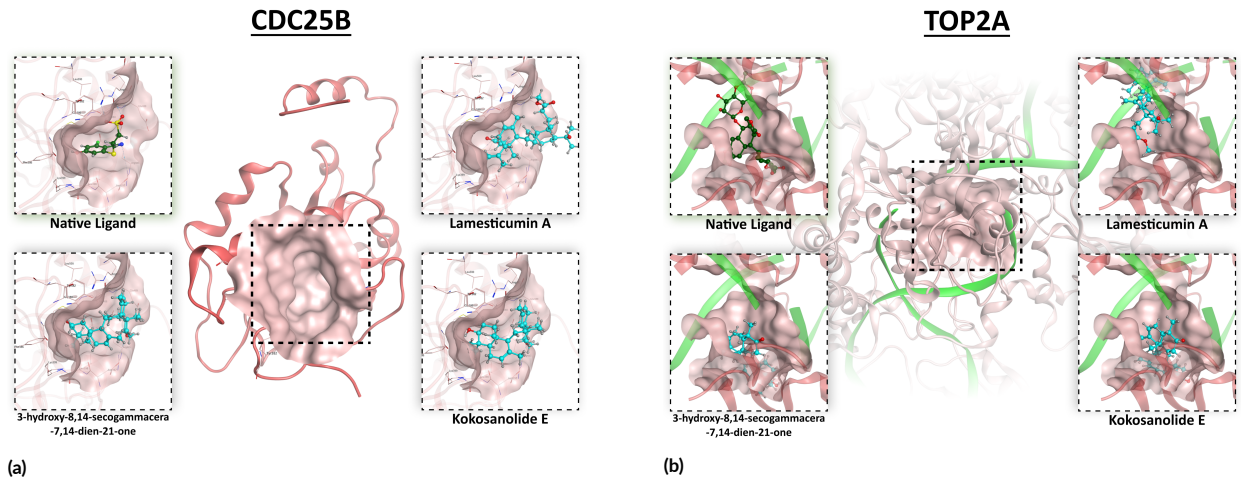


FIGURE 4 Potential protein targets of DPE compounds. The InteractiVenn showed that CDC25B and TOP2A as potential targets of DPE.



(a) (b)

	CDC25B (ID:4WH9)			TOP2A (ID:5GWK)		
	Docking Score (ΔG)	RMSD (Å)	Amino Acid Residues	Docking Score (ΔG)	RMSD (Å)	Amino Acid Residues
Native Ligand	-5.6039	0.3036	Tyr 382, Phe 386, Asp 397, Leu 398, Cys 484, Arg 485, Arg 488, Arg 492, Met 505	-10.5635	1.4407	Glu A461, Gly A462, Asp A463, Leu A486, Arg A487, Gly A488, Met A762, Met A765, Met A766, Pro A803
3-Hydroxy-8,14- secogammacera-7,14- dien-21-one	-6.6665	-	Glu 377, Leu 378, Ile 379, Gly 380, Tyr 382, Phe 386, Asp 397, Lys 399, Arg 485, Arg 488, Glu 489, Arg 492, Pro 503	-7.8087	-	Lys A440, Asp A463, Arg A487, Gly A488
Lamesticumin A	-6.2781	-	Glu 377, Asp 397, Tyr 382, Leu 398, Lys 399, Cys, 484, Arg 485, Arg 488, Glu 489, Arg 492, met 505	-8.5704	-	Met A762, Met A765, Met A766, Ser A802, Pro A803, Arg A804
Kokosanolide E	-6.1492	-	Glu 377, Leu 378, Ile 379, Gly 380, Tyr 382, Phe 386, Asp 397, Lys 399, Arg 485, Arg 488, Glu 489, Arg 492, Pro 503	-7.2297	-	Lys A440, Asp A463, Arg A487, Gly A488

(c)

FIGURE 5 Model of protein-molecule interaction. DPE compounds including 3-hydroxy-8,14-secogammacera-7,14-dien-21-one, lamesticumin A, and kokosanolide E bind to CDC25B (a) and TOP2A (b) compared to their respective native ligand, suggesting their role as inhibitor. The docking score in the targeted protein were compared for their binding affinities and amino acid residues (c).

3.5. Discussion

This study aims to investigate the potential of DPE to be developed as an adjunctive agent of Dox against triple negative breast cancer (TNBC) cell model, 4T1. TNBC is one of the subtypes of breast cancer characterized by treatment limitation and poor efficacy (Yin et al. 2020). The 4T1 cells are suitable for this experiment because of their active proliferation and migration characteristics (Kaur et al. 2012). Chemotherapy is still the first choice to treat TNBC, however, unfortunately chemotherapy can affect the resistance for a long time (Hermawan et al. 2021). Accordingly, adjunctive treatment is one of the solutions to overcome the effectiveness of chemotherapy.

First, we examined the single cytotoxic effects of DPE on 4T1 cells. The previous studies reported that isolate of DPE has moderate cytotoxic activity on breast cancer cells. The IC_{50} value of DPE compounds are 30.69 µg/mL for 3-hydroxy-8,14-secogammacera-7,14-dien-21-one, 15.68 µg/mL for Lamesticumin A, and 45.90 µg/mL for Kokosanolide E (Fadhilah et al. 2021; Labibah et al. 2021; Mayanti et al. 2023). Our study revealed that DPE has cytotoxic activity with an IC_{50} value of 47 µg/mL. Interestingly, the IC_{50} value from DPE crude extract in this study was close to the IC_{50} value of the isolated compounds. This suggests that the active compounds in the crude extract may be present in sufficient concentration and/or work synergistically. Bioinformatics analysis indicates that DPE may potentially target the overexpressed CDC25B. CDC25B plays a main role in the initial activation of CDK-1-cyclin B during G2-M transition, suggesting that the cytotoxic mechanism of DPE may involve the by inhibition in the G2-M transition (Boutros et al. 2007). This hypothesis requires further evidence, warranting additional cell cycle analysis.

Doxorubicin is one of the most common first line cancer therapies, including breast cancer with TNBC subtypes (Ikawati et al. 2020). In this study, we used Dox as a representative chemotherapeutic agent. Interestingly, when combined with Dox, DPE exhibited a promising synergistic effect indicate by CI value less than 1 (0.34) (Chou 2010). The observed synergistic effect potentially from complementary mechanisms of action; however, further analysis is required to confirm this hypothesis. Furthermore, DPE showed significant inhibition of cells migration, surpassing the effect of DPE. Targeting cell migration will be a good prospect in developing treatment for TNBC due to its high rate of metastasis (Singh and Yadav 2021). The potential targets of DPE identified through bioinformatics approaches were TOP2A and CDC25B. TOP2A over expression may promote the proliferation, metastasis, and invasion of cancer cells, whereas CDC25B silencing could suppress the invasion and migration of gastric cancer (Leal et al. 2016; Wang et al. 2022). Although DPE exhibited stronger binding to CDC25B but weaker binding to TOP2A than of its native ligand (the established inhibitor in the crystal structure), its migration assay demonstrated significant inhibition compared to control and Dox at 24 µg/mL. In this regard, the anti-migratory mechanism of DPE may involve the same binding site as the native ligands of the CDC25B protein used in molecular docking, or it may operate through a different mechanism in inhibiting migration. This suggests further investigation about the mechanisms underlying these synergistic effects. Nevertheless, this result suggests that DPE has potential for enhancing the efficacy of chemotherapeutic agents.

Overall, DPE is promising to be developed as an adjunctive treatment for TNBC. In this study, DPE has cytotoxic activity and has good synergistic effects with Dox against TNBC cells. The challenge of future studies in deepening the exploration of active compounds in DPE. Furthermore, the next studies can investigate the cytotoxicity in other cancer cell lines and the related molecular mechanism. In addition, cytotoxic tests on normal cells are needed to prove the selectivity of DPE against cancer cells.

4. Conclusions

This study concluded that DPE showed moderate cytotoxic activity against 4T1 cells as a model of TNBC. The combination of DPE with Dox showed synergistic and anti-migration effect. Additionally, the bioinformatics approaches showed that DPE potentially targeted CDC25B and TOP2A, suggesting that DPE have the potential to be adjunctive treatment of Doxorubicin in TNBC.

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Authors' contributions

FAR, NPH wrote the manuscript. FAR carried out virtual screening and analyzed the data. ANS, DRR, NPH carried out and analyzed the in vitro work. EM, RAS supervised and designed the experiment and the manuscript.

Competing interests

There were no conflicts of interest in this article.

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