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Original Research Article

Peperomia pellucida Extract Enhances the Cytotoxicity of Doxorubicin against 4T1 Breast Cancer Cells

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ABSTRACT

The use of natural agents in co-chemotherapy to enhance activity while minimizing adverse effect has been greatly explored. Doxorubicin, one of the chemotherapeutic drugs for breast cancer, exhibits serious adverse effects on the normal cells. *Peperomia pellucida* has potential as an anticancer and antioxidant agent. The present study aimed to investigate the combined effect of *Peperomia pellucida* extract (PPE) and doxorubicin on triple-negative breast cancer (TNBC) cells (4T1). PPE was obtained by maceration in 96% ethanol. The cytotoxic activity was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. PPE exhibited moderate cytotoxic effect against 4T1 cells with IC_{50} value of 83.86 µg/mL. PPE showed synergistic activity when combined with doxorubicin (IC_{50} = 0.4 µg/mL) and was less toxic to Vero cell lines (IC_{50} = 145.36 µg/mL). The findings from this study provide credence to the idea of using PPE in conjunction with doxorubicin as a chemotherapeutic agent to treat TNBC.

Keywords: *Peperomia pellucida*, Breast cancer, Co-chemotherapy, Combination index, Doxorubicin.

Introduction

Doxorubicin is a widely used chemotherapeutic agent for killing cancer cells and inhibiting their growth.[1] However, it possessed several adverse effects such as cardiotoxicity and nephrotoxicity due to its unselective activity.[2] The use of doxorubicin can cause harm to normal cells and induced cell senescence.[3] Combination treatment or co-chemotherapy with other drug is necessary to minimize the occurrence of side effects along with maintaining or increasing anticancer activity.[4] Natural agents have the potential to be developed as co-chemotherapeutic agent to treat cancer.[5] Natural ingredients, such as plant extract, have shown potential in the discovery of anticancer agent.[6] A recent study reported that galangal extracts synergistically improve the cytotoxic effect of doxorubicin on 4T1 cells and simultaneously reduce the incidence of cellular senescence on NIH-3T3 fibroblast cells.[4] Rice bran extract combined with doxorubicin also demonstrates this effect in treating 4T1 cells.[7] Both extracts show similar mechanisms through increasing reactive oxygen species (ROS) levels over the normal threshold, specifically in cancer cells. These two natural agents have shown that developing co-chemotherapeutic agents is a promising strategy to fight cancer. However, further study is still needed to identify agents that have greater potential, more accessible, and selectively target cancer cells. *Peperomia pellucida* commonly known as "suruhan" or "ketumpang air" in Indonesia,[8] is an easy to find plant and is widely spread in almost all types of habitats. Researchers have studied *Peperomia pellucida* for its antibacterial, anti-inflammatory, analgesic, antifungal, and anticancer properties.[9–14] The anticancer activity of *Peperomia pellucida* extract (PPE) has been explored *in vitro* against MCF7 breast cancer, HeLa cervical cancer, and HepG2 hepatic carcinoma cells.[9,15] In this study, the 4T1 breast cancer cell line was employed as a cancer model for testing the anticancer attributes of PPE alone and in combination with doxorubicin. Vero cell line was used as a non-malignant model for assessing the selectivity index (SI). The aim of this study was to assess the efficacy of PPE as a co-chemotherapeutic medication against triple-negative breast cancer (TNBC). The results will provide extensive new confirmation of PPE as a potential chemotherapeutic agent against TNBC.

Materials and Methods

Cell lines and culture

4T1 (ATCC, ref. # HTB-22) and Vero (ATCC, ref. # CCL81.2) cells were obtained from the Laboratorium of Molecular Biology, Research Center for Medicinal Raw Materials and Traditional Medicine, Tawangmangu, Indonesia. The cells were sustained in Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Sigma), 1% penicillin-streptomycin (Gibco), sodium bicarbonate (Sigma), and HEPES (Sigma).

Plant collection and identification

Peperomia pellucida whole plants were collected in November 2024 from Maguwoharjo, Sleman, Yogyakarta (location coordinate: 7°46'01"S 110°25'58"E). The plants were identified by Siti Kartikasari, Laboratorium of Biology, Faculty of Teacher Training and Education, Universitas Muhammadiyah Surakarta. The plants were deposited in Laboratorium of Pharmacology and Toxicology, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta, and voucher number 030/A-E-I/LAB.BIO/IX/2023 was issued.

Plant extraction

The *Peperomia pellucida* whole plants were dried and crushed into powder. The powdered plant material was extracted by maceration in 96% ethanol at room temperature for 48 h. The extract was filtered, and

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the residue was re-macerated in ethanol for 24 h.[8,13] The combined extract was concentrated in a rotary evaporator at reduced pressure, resulting in a dried *Peperomia pellucida* ethanol extract (PPE).

Phytochemical screening
Preliminary phytochemical screening of PPE was done to detect the presence of phytochemicals such as polyphenols, alkaloids, flavonoids, anthraquinones, tannins, and saponin according to standard method.[16]

MTT assay
The 4T1 and Vero cells were seeded in a 96-well plate at a density of 7 × 10^3 cells per well and treated with serial concentrations of PPE (1 – 200 µg/mL) and doxorubicin (0.1 – 6 µg/mL) as single agent and then in combination. The cells were incubated at 37ºC for 24 h in a 5% CO_2 atmosphere. After treatment, the cells were washed with phosphate buffered saline (PBS), then 100 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (0.5 mg/mL) in DMEM was added to each well and then incubated at 37ºC for 3 h. The reaction of MTT formazan was stopped by the addition of 10% sodium dodecyl sulfate in 0.01 N HCl, followed by incubation at room temperature overnight. The absorbance of the resulting solution was measured at 595 nm using a microplate reader.[17,18] The concentrations that inhibit 50% of cell viability (IC_{50}) were calculated and used for assessing the cytotoxic activity of the combined agents, and then the combination index (CI) was determined. The IC_{50} values were also used to determine the selectivity index (SI) by calculating the ratio of the IC_{50} values for cancer cells and normal cells.[19] The synergistic effect of PPE and doxorubicin was interpreted by the CI score based on the cell viability.[20]

Statistical analysis
Data were analyzed using Microsoft Excel 2010 and presented as the mean ± standard error of the mean (SEM), n = 3. Differences between means were analyzed by one-way analysis of variance followed by Tukey's post hoc test using SPSS version 18. A p-value of <0.05 was considered statistically significant.

Results and Discussion

Peperomia pellucida locally known as "ketumpangan air/suruhan" in Indonesia. It is claimed by the local community as a herbal medicine for the treatment of various diseases.[8] The plant contains various phytochemicals, such as polyphenols and flavonoids, that contributes to its pharmacological effect.[11,12] *Peperomia pellucida* is considered a possible chemotherapeutic agent that can be used in combination with doxorubicin for the treatment of metastatic breast cancer. This study provides a valuable perspective for the development of alternative agents for breast cancer, and reduce the over dependent on the use of doxorubicin, thereby resulting in the reduction of doxorubicin dose, and mitigation of its adverse effects.

Percentage yield and phytochemical constituents of Peperomia pellucida extract (PPE)
The percentage yield of PPE was 17.70%. Phytochemical screening indicated the presence of polyphenols, flavonoids, anthraquinones, and saponins (Table 1). This result is a preliminary data upon which further investigation on the phytoconstituents of PPE will be conducted.[21]

Table 1: Phytochemical constituents of *Peperomia pellucida* ethanol extract

Phytochemical constituent	Type of test	Inference
Polyphenols	Ferric chloride ($FeCl_3$)	+
Alkaloids	Dragendorff	-
Anthraquinones	$FeCl_3$, Chloroform, NaOH	+
Tannins	NaCl (2%) and Gelatin	-
Saponins	Frothing	+
Flavonoids	UV (366 nm)	+

+: Indicate present; -: Indicate absent

Cytotoxicity of PPE
PPE demonstrated a concentration-dependent cytotoxic effect against 4T1 cells, with IC_{50} value of 83.86 µg/mL (Figure 1A). In comparison, doxorubicin demonstrated a more potent cytotoxic effect, with an IC_{50} value of 0.37 µg/mL (Figure 1B). Based on the IC_{50} value, PPE can be said to have moderate cytotoxic effect against 4T1 breast cancer cells.[22] The cytotoxic activity of PPE and doxorubicin on Vero cells, which represented the normal cell model was also investigated. The results showed a higher IC_{50} values for both PPE and doxorubicin (Figure 2). The IC_{50} values of PPE and doxorubicin against Vero cells were 145.36 and 4.08 µg/mL, respectively. The selectivity index (SI) was determined by comparing the IC_{50} value in normal cells and the IC_{50} in cancer cells. Selectivity is an important property of desired chemotherapeutic agents, in which a compound should exhibit higher potency against cancer cells compared to normal cells.[19]

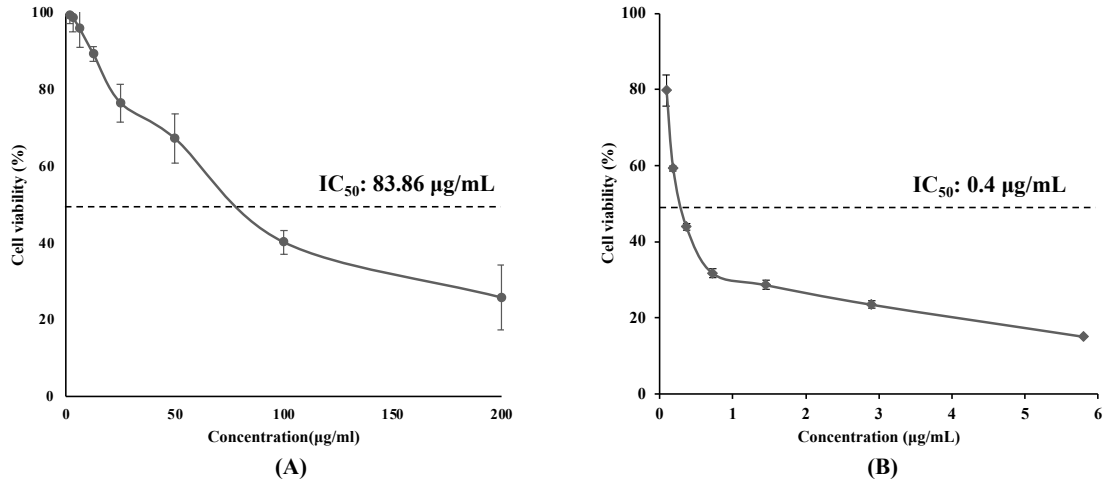

Figure 1: Cytotoxic activity of PPE (A) and doxorubicin (B) against 4T1 cells. The data represent mean ± standard error of mean (SEM) (n = 3), and the dashed line represent the 50% cell viability

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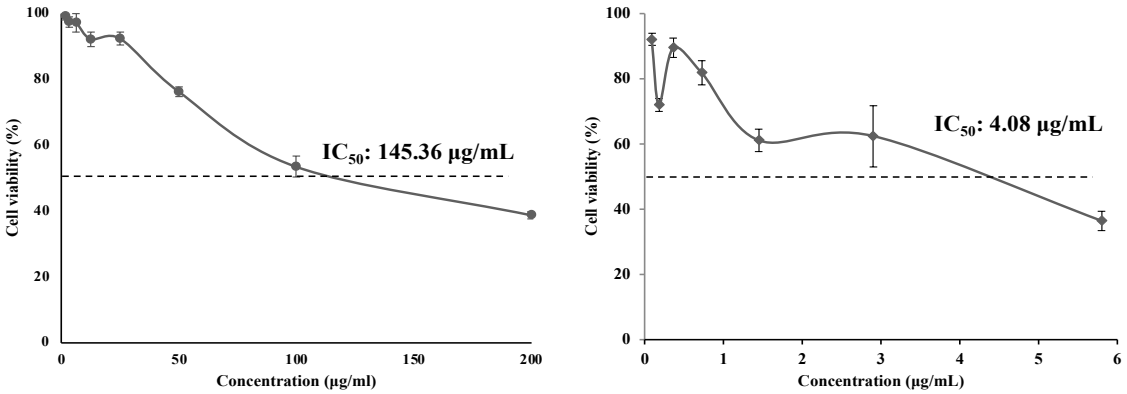


(A) **(B)**

Figure 2: Cytotoxic activity of PPE (A) and doxorubicin (B) on Vero cells. The data represent mean ± standard error of mean (SEM) (n = 3), and the dashed line represent the 50% cell viability

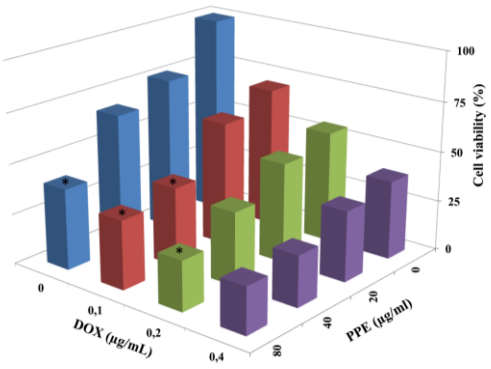
Generally, the SI value of a selective anticancer compound should be greater than 3.[23] In this study, the SI value of PPE was 1.73, hence it is considered non-selective to cancer cells (Table 2). Compared to other plant extracts such as galanga extract that have been used in combination with doxorubicin against 4T1 breast cancer cells,[4,24] PPE exhibited more potent effect. As a natural agent, PPE with an IC_{50} below 100 µg/mL warrants further investigation on other cancer cell types, and to identify the active ingredient.

Cytotoxic activity of the combination of PPE and Doxorubicin

The combination of PPE and doxorubicin showed strong synergistic cytotoxic effects against 4T1 breast cancer cells, with CI score ranging from 0.01 to 0.67 (Figure 3). CI score between 0.3 - 0.7 indicates synergistic effect, while CI score of 0.3 - 0.1 indicate a strong synergistic effect.[20,25,26] In summary, PPE alone showed moderate cytotoxic activity against 4T1 cells, but showed more potent cytotoxic effect when combined with doxorubicin. This observation is in line with previous studies where the combination of plant extracts with doxorubicin were found to exhibit greater cytotoxic effect against 4T1 breast cancer cells compared to using the individual extract alone.[4,25,27,28]

Table 2: Cytotoxic activity and Selectivity Index (SI) of PPE against 4T1 and Vero cells

Sample	IC_{50} (µg/mL)		SI
	4T1	Vero	
PPE	83.86	145.08	1.73
Doxorubicin	0.4	4.08	10.2



D0X (µg/mL)	PPE ((µg/mL)		
	20	40	80
0,1	0,15	0,37	0,67
0,2	0,41	0,20	0,06
0,4	0,18	0,05	0,01

(A) **(B)**

Figure 3: Cytotoxic activity of the combination of PPE and doxorubicin against 4T1 cells (A) and the Combination Index (B). The cells were cultured in a 96-well plate at a density of 7×10^3 cells/well. The treatment was performed at sub-doses of the IC_{50} (20, 40, and 80 µg/mL for PPE) and (0.1, 0.2, and 0.4 µg/mL for doxorubicin) for 24 h. The cell's viability was assessed by performing MTT assay. (*$p<0.05$, compared to single dose of PPE)

Further research is necessary to determine the ideal concentration of both substances that will inhibit cancer cell growth while preserving the normal cells. Other investigations such as anti-migratory activity using the scratch wound healing assay and gelatin zymography[29–32] may also be necessary to complement the data on cytotoxic activity of PPE against highly metastatic breast cancer cells. Moreover, the use of additional cancer model such as human TNBC cells is needed for more comprehensive data that would be useful in the development of breast cancer co-chemotherapeutic candidates.

Conclusion

Peperomia pellucida ethanol extract (PPE) has been shown to exhibit moderate cytotoxicity toward 4T1 breast cancer cells. PPE significantly enhanced the cytotoxic effect of doxorubicin in 4T1 cells, resulting in a strong synergistic effect. The potential of PPE as a co-chemotherapy agent with doxorubicin needs further investigation.

Conflict of interest

The author reports no conflicts of interest in this work.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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