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Original Research Article

Synergistic Effects of the Combination of Active Fractions of Red Ginger Rhizome (*Zingiber officinale var. rubrum*) with Doxorubicin on 4T1 Cells

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ABSTRACT

The use of chemopreventive agents in combination with chemotherapy increases the sensitivity of cancer cells, enhances the efficacy of chemotherapy, and reduces damage to healthy tissues. Red ginger rhizomes are among the natural materials that could be created as co-chemotherapy agents. The research aims to evaluate the effect of combining active fractions from red ginger rhizome with doxorubicin on the inhibition of 4T1 breast cancer cell proliferation *in vitro*. The extract of red ginger rhizome was obtained by maceration in 96% ethanol and fractionated using n-hexane, ethyl acetate, and a water-methanol mixture. The cytotoxicity effect was assessed using the MTT test, while proliferation inhibition and apoptosis were evaluated using the doubling time and AO/PI staining methods. The results showed that n-hexane fraction exhibited a more effective cytotoxic effect, with an IC_{50} value of 26 µg/mL, in contrast to the ethyl acetate and water-methanol fractions, which had IC_{50} values of 72 µg/mL and 89 µg/mL, respectively. The n-hexane fraction has a very strong synergistic effect when combined with doxorubicin, with a Combination Index (CI) value of < 0.1. Combination (n-hexane fraction 1/8 IC_{50} with doxorubicin 1/4 IC_{50}) can extend the doubling time, and the combination (n-hexane fraction ½ IC_{50}; doxorubicin ½ IC_{50}) can induce apoptosis. The findings of this study suggest that the n-hexane fraction of red ginger rhizome has potential as a co-chemotherapy agent in combination with doxorubicin against 4T1 breast cancer cells.

Keywords: Red ginger (*Zingiber officinale var. rubrum*), 4T1 breast cancer, doxorubicin, co-chemotherapy

Introduction

Worldwide, cancer has become the biggest killer.[1] A total of 396,914 instances of breast cancer were reported in 2020 based on data from the Global Cancer Observatory (GLOBOCAN).[2] Worldwide, 28.4 million new instances of breast cancer would be reported by 2040, with an annual death toll of 1 million, per the World Health Organization (WHO).[3,4,5] The characteristic of the triple-negative breast cancer (TNBC) subtype is the absence of estrogen receptors (ER), epidermal growth factor receptor-2 (HER-2), and progesterone receptors (PR).[6] Chemotherapy remains the primary option for 4T1 breast cancer cells due to limited therapy choices.[1,7] One of the most widely used chemotherapy medications for breast cancer treatment is doxorubicin.[8] However, doxorubicin has several issues including toxicity to normal tissues, acute adverse effects, and the emergence of resistance.[9] These side effects occur because doxorubicin targets DNA that is also present in normal cells, so normal cells are affected by these side effects.[10] One potential strategy is the use of co-chemotherapy, which combines chemotherapy medicines with chemopreventive chemicals that are less toxic or non-toxic to lessen the treatment's harmful effects on healthy tissues while increasing its efficacy.[11,12]

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One way to discover chemopreventive compounds is by exploring natural materials.[13] Indonesia is a country rich in plant-based natural medicines that the community has used for several generations to treat various diseases, including cancer.[7,14] Researchers found that galangal extract reduces cellular aging in NIH-3T3 fibroblast cells and synergistically enhances the cytotoxic effects of doxorubicin on 4T1 cells.[15] In 4T1 breast cancer cells, it has the same effect when rice bran extract was combined with doxorubicin.[16] These two natural agents have shown that the development of co-chemotherapy agents is a promising strategy for combating cancer. Red ginger rhizome is known to have superior benefits, such as its antiemetic effect, which is greatly needed in cancer treatment.[17] Red ginger rhizome is recognized for plenty of advantages, including its antiemetic properties, which are particularly beneficial in cancer therapy. 6-gingerol and 6-shogaol, two principal compounds found in red ginger rhizome, exhibit anticancer effects that can inhibit the growth of breast cancer cells. 10-gingerol exhibits a far potent anticancer effect than 6-gingerol and 6-shogaol, owing to its anti-neuroinflammatory properties, which potentially reduce metastasis.[18,19] Researchers have investigated the potential anticancer effects of red ginger rhizome extract using HeLa and T47D cells.[20,21] In 4T1 breast cancer cells, there have been no scientific reports on the fractions of red ginger rhizome as a chemopreventive agent. This study was conducted to evaluate the combined effects of the active fraction of red ginger rhizome and doxorubicin in suppressing the progression of 4T1 breast cancer cells *in vitro*.

Materials and Methods

Red Ginger Rhizome Ingredients

The powder from the rhizome of red ginger was obtained from the Indonesian Institute of Medicinal (BPTO) in Tawangmangu, Jawa Tengah, Indonesia. The simplicia powder was macerated using a 96% ethanol solvent. The resulting extract was then concentrated using a rotary evaporator (Heidolph, Germany) and maintained at a temperature of 45°C with a water bath. The extract was partitioned using a liquid-liquid method with n-hexane, ethyl acetate, and a water-methanol

mixture. The fraction was used as samples for single cytotoxicity tests. Further research was conducted on samples that showed potential for combination cytotoxicity tests, cell proliferation inhibition, and apoptosis observation.

Cell Lines and Culture

Following their cultivation in an incubator with 5% CO_2, the 4T1 breast cancer cells (ATCC, ref. #HTB-22) reached confluence. These cells were cultured in a medium which comprised 10% fetal bovine serum (FBS) (Sigma), 100% glucose, and 1% penicillin and streptomycin from Gibco, USA.

MTT assay

At a density of 1 x 10^4 cells/well, 4T1 cells were cultured in a 96-well plate until they reached 80% confluence. Subsequently, the cells were treated with doxorubicin (5; 2.5; 1.25; 0.6; 0.3; 0.16 µg/mL) and red ginger rhizome fraction (50; 100; 200; 400; 800 µg/mL). Meanwhile, concentrations used in the combination below the IC_{50} value were carried out using the same procedure as the combination cytotoxicity test. After incubation, the cells were washed with PBS (Sigma), and MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was added. Reincubated at 37˚C for 2 to 4 hours and then stored at room temperature overnight after adding a 10% SDS solution in 0.01 M HCl. At 595 nm, an ELISA reader (Biorad, USA) was used to assess absorbance. Cell viability percentages were then calculated using absorbance data from single and combination treatments to obtain the IC_{50} value for single treatments and the combination index (CI) for a combination treatment of doxorubicin and the active fraction of red ginger rhizome.[22]

Inhibition of cell proliferation (Doubling time)

Cell proliferation inhibition was carried out using the MTT method. The samples used were at concentrations below IC_{50} (1/2; 1/4 IC_{50}). Observations were made at 24, 48, and 72 hours.[23] The absorbance results obtained using the ELISA reader are used to determine cell viability. The incubation time is plotted against the living cells. The doubling time was determined using the following equation.[2,24,25]

$$Doubling\ \ time = \frac{Y-A}{B} + 100\% \ldots (\text{eq. } 1)$$

Y: log (2 x initial number of living cells)
A: Intersect
B: slope

Apoptosis test

Acridine Orange/Propidium Iodide (PI) reagent is used to analyze apoptosis. In a 5% CO_2 incubator, cells were cultured on coverslips in 24-well plates at a density of 2 × 10^{x5} cells/well until they reached confluence. The cells were treated for 24 hours with doxorubicin and the active component of red ginger rhizome. After that, the coverslip was placed on a microscope slide and stained with 10 µL of the AO/PI reagent mixture, which is prepared by dissolving AO and PI (10 µg/mL) (Sigma-Aldrich, St. Louis, MO, USA) in PBS (Sigma). Look through a Leica Microsystem fluorescent microscope at Wetzlar, Germany.[24]

Statistical analysis

The cytotoxic effect of the extract was determined by converting the ELISA readings into the percentage of live cells to calculate the IC_{50} values. Data is presented as the mean ± SD of three repetitions. The data on cell proliferation inhibition were analyzed using GraphPad Prism 10 (GraphPad Software, USA) and one-way ANOVA. A p-value ≤ 0.05 indicates a statistically significant difference. The doubling time was calculated by comparing the incubation time with the number of living cells.

Results and Discussion

This study aims to evaluate the cytotoxic effects of the plant extracts both individually and in combination with doxorubicin. Therefore, to ensure that the cells are alive, we used the MTT assay, which involves the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) compound. An insoluble purple formazan, which can dissolve in 10% SDS, is produced by living cells that convert MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) molecules. This process is the basis of the MTT method. This transformation is carried out by utilizing the enzyme succinate-tetrazolium reductase in the mitochondria.[26] The IC_{50} values for the n-hexane, ethyl acetate, and water-methanol fractions were found to be 26 µg/mL, 72 µg/mL, and 89 µg/mL, respectively, against the 4T1 cells (Figure 1). Based on the IC_{50} values, it is categorized as having a fairly active cytotoxic effect.[27] However, this fraction of n-hexane demonstrated superior suppression of 4T1 cell growth. In comparison, doxorubicin exhibited a more potent cytotoxic effect, as seen in Figure 1, exhibiting an IC_{50} value of 0.4 µg/mL. Our findings align with prior studies on HeLa cancer cells, where the n-hexane fraction is known to have significant cytotoxic activity, exhibiting an IC_{50} value of 20.350 µg/mL.[28] In addition, the IC_{50} value for the red ginger ethanol extract is 22.39 µg/mL, indicating that the extract can kill T47D breast cancer cells.[21] Therefore, this is worth further investigation as a potential anticancer candidate for 4T1 breast cancer. Alterations in cell morphology and diminished cell viability are characteristic of cytotoxic effects. Cells that were still alive after sample treatment show signs of cell shrinkage (Figure 2).

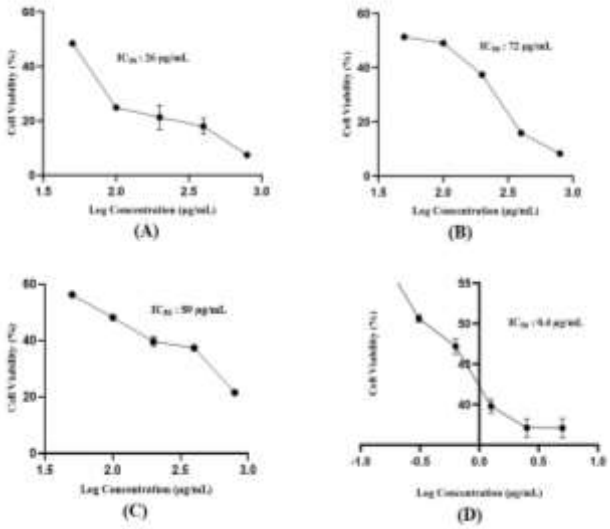

Figure 1: Cytotoxic activity of ZOVR (A) n-hexane fraction, (B) ethyl acetate fraction, (C) water-methanol fraction, and (C) doxorubicin against 4T1 breast cancer cells. The cytotoxic effect was measured using the MTT assay over 24 hours. The displayed values represent the mean ± SD (N = 3).

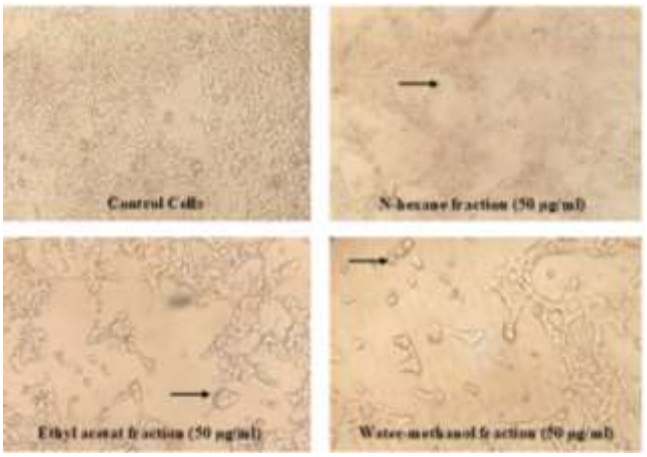

Figure 2: Changes in the morphology of 4T1 breast cancer cells after treatment with various fractions of red ginger rhizome were observed using an inverted microscope at 100x magnification. The morphological changes in 4T1 cells are indicated by arrows.

The features of cell death, or apoptosis, encompass chromatin condensation, the result of plasma membrane enlargement, damaged DNA, and the fragmentation of cells into minute fragments known as apoptotic bodies.[29] Furthermore, the cytotoxic effects of the active fraction of red ginger rhizome combined with doxorubicin on 4T1 cells will be evaluated in this study. Combining the n-hexane fraction of ZOVR (*Zingiber officinale var. rubrum*) with doxorubicin resulted in a decrease in percentage cell survival due to its cytotoxic effects, as shown in Figure 3A, compared to the treatment with doxorubicin alone. Our combination shows a very strong synergistic effect, with a CI score that varies from 0.0 to 0.02 (Figure 3B). A CI score of less than 0.1 indicates a potent synergistic effect, while a value between 0.3 and 0.7 signifies a robust synergistic effect.[30] Therefore, a combination with n-hexane fraction and doxorubicin can augment the cytotoxic effects on 4T1 cells. Previous studies have shown that doxorubicin combined with plant extracts, such as *Caesalpinia sappan* and *Ficus septic*, *Alpinia galanga* L., and *Hibiscus Sabdariffa*, has greater cytotoxic effects on 4T1 breast cancer cells compared to when used alone. Thus, our findings are consistent with this.[11,15,25]

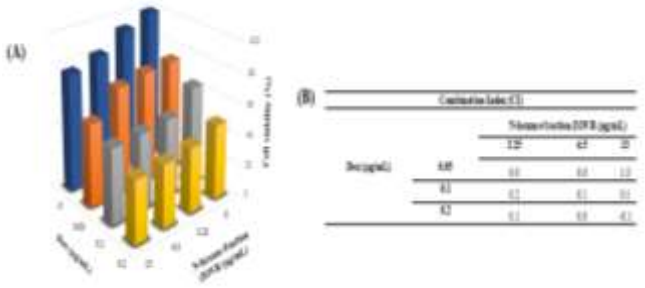

Figure 3: Profile of 4T1 cell growth inhibition after treatment with a combination of ZOVR n-hexane fraction (3.25; 6.5; 13 µg/mL) and doxorubicin (0.05; 0.1; 0.2 µg/mL) (A) and combination index values (B). Cell viability was evaluated using the MTT assay for all treatments over 24 hours, as described in the methods. Dox: doxorubicin

Cell proliferation inhibition testing was also conducted using the MTT method, both individually and in combination with doxorubicin. The concentrations tested were 1/4 and 1/8 of the IC_{50}. It is expected that at concentrations lower than the IC_{50} value, not many cells will die during the 72-hour observation due to the cytotoxic nature of the test compound.[5] Absorbance data show the quantity of living cells and illustrate the ability of the sample to inhibit growth of cells at 24, 48, and 72 hours. At each incubation time (24, 48, and 72 hours), Figure 4A clearly shows that the control group, single treatment, and combination of n-hexane fraction 1/8 IC_{50} and Dox 1/4 IC_{50} exhibit significantly different cell viability ($p < 0.05$). The inhibitory effect in the combination treatment was much higher compared to the single treatment. This is comparable to the cell doubling time value of a combination treatment with the fraction of n-hexane 1/8 IC_{50} with doxorubicin 1/4 IC_{50}, which was much higher than the single treatment, as can be seen in (Figure 4B). Compounds that slow down cell doubling time are considered to inhibit genes or protein chains that are involved in the cell cycle.[31] A doubling of time is a duration necessary for a cell to replicate its quantity.[32] The data above shows that the combination of n-hexane fraction treatment with doxorubicin does not stop the cell cycle, but rather likely only causes cell cycle arrest, thereby impeding cell development. The bioactive components contained in red ginger's rhizome, such as 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol, were shown in prior research to induce cell cycle cessation and cell apoptosis.[33] Active compounds such as 6-shogaol from ginger (*Zingiber officinale*) are capable of providing antiproliferative cell activity by downregulating Notch signaling (Hes1 and Cyclin D1 genes), which induces a significant accumulation of the cells cycle dependent on the G2/M phase in breast cancer cells.[34] 6-Gingerol compound induces apoptosis in colorectal cancer cells and halts the cell's cycle during the G1 phase, both of which limit cell growth.[35] 10-gingerol compound can inactivate the transcription factors Akt enzyme and p38 protein MAPK, which subsequently impede the growth and dissemination of breast cancer cells.

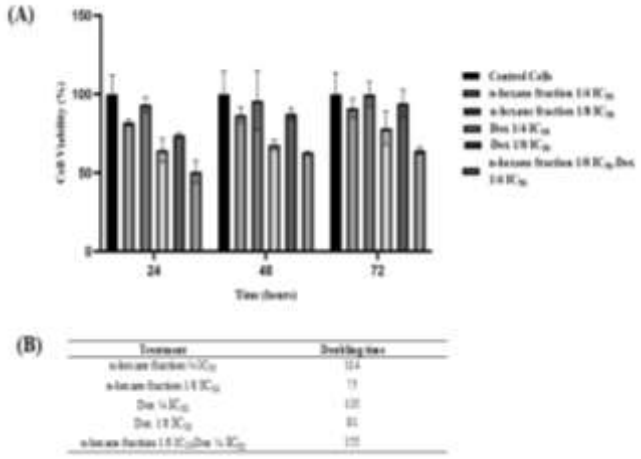

Figure 4: The effect of each single treatment and the combination of n-hexane fraction with dox on the proliferation of 4T1 breast cancer cells. (A) Inhibition of 4T1 cell proliferation after single and combination treatments with concentrations below IC_{50} (1/4 and 1/8) over incubation periods of 24, 48, and 72 hours. (B) The doubling time values for each sample treatment. Data are presented as mean ± SD (N=3), with significance ($P < 0.05$).

This diminishes EGFR expression in ER-negative MCF-7 and ER-positive (MDA-MB-231) cell lines.[36] Fluorescence microscopy can identify several forms of cell death, including apoptosis, which is a physiological mechanism of programmed cell death with therapeutic potential in cancer.[37] Apoptosis is characterized by the occurrence of blebbing on the cell membrane without losing its integrity, cytoplasmic shrinkage, nuclear condensation, and fragmentation into apoptotic bodies.[31] Cell apoptosis observation was conducted using staining with the orange acridine-propidium iodide reagent. Orange acridine can pass through the membranes of both live and dead cells, where it binds to double-stranded DNA, emitting green fluorescence, and to single-stranded DNA, emitting orange fluorescence. While propidium iodide (PI) cannot penetrate healthy cells, it can enter cells with disrupted plasma membranes due to necrosis, resulting in red fluorescence as a consequence of its interaction with cellular nucleic acids. This dye can only be absorbed by cells with disrupted membranes.[38] The formation of red fluorescence indicates that the cells are undergoing apoptosis at the late stage, that was the cell demise cycle.[37] The research results show that the control cells (Figure 5a) exhibited green fluorescence with intact nuclear morphology. The early stages of apoptosis were observed in (Figure 5b), characterized by yellow-green fluorescence and nuclear condensation due to the administration of doxorubicin (0.2 µg/mL). Additionally, treatment with the n-hexane fraction and its combination with doxorubicin can induce apoptosis, characterized by nuclear loss, membrane swelling, and the presence of dead cells with red fluorescence (Figures 5c and d). This combination makes the n-hexane fraction at ½ IC_{50} capable of enhancing the cytotoxic effects of doxorubicin, causing 4T1 breast cancer cells to undergo cell death. Research conducted by Ekowati (2012) supports the finding that a mixture with Java chili (*Piper retrofractum*) and red ginger extract can induce apoptosis in Myeloma and Widr cells through p53 expression.[39] Previous studies have shown that ginger, scientifically known as *Zingiber officinale*, can induce cell death by activating caspase-3 and releasing cytochrome C through the mitochondrial pathway, similar to curcumin.[39] The active constituents in red ginger, including 6-gingerol, can induce apoptosis by elevating the Bax/Bcl-3 ratio, releasing cytochrome C, mactivating the AMPK cascade, and inhibiting PI3/AKT and mTOR pathways.[40,41]

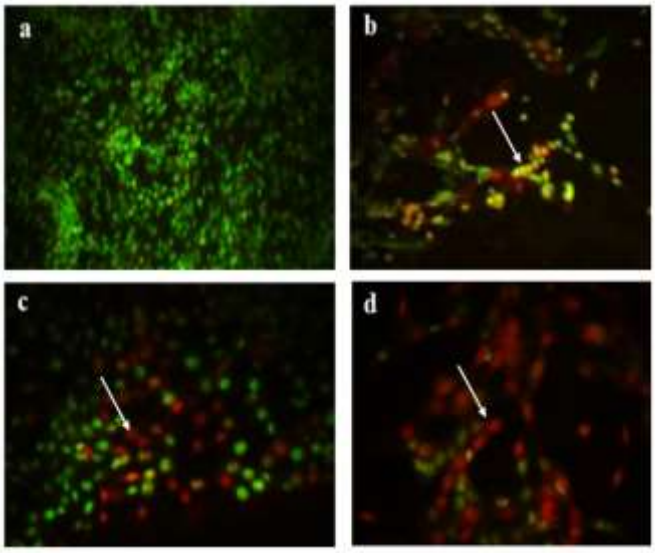

Figure 5: Observation of 4T1 cell morphology after double staining using propidium iodide-acridine orange as described in the method, viewed under a 400x fluorescence microscope. Control cells (a), doxorubicin ½ IC_{50} (0.2 µg/mL) (b), n-hexane fraction treatment ½ IC_{50} (13 µg/mL) (c), and combination (n-hexane fraction ½ IC_{50}; doxorubicin ½ IC_{50}) (d). In the control cells, living cells fluoresce green. Apoptotic cells have fragmented cells (red fluorescence).

Conclusion

The n-hexane fraction of red ginger rhizome has cytotoxic effects against 4T1 cell lines with an IC_{50} value of 26 µg/mL. The combination of the n-hexane fraction and doxorubicin produces a very strong synergistic effect in inhibiting cell proliferation and can induce apoptosis. The n-hexane fraction, as a co-chemotherapy agent with doxorubicin, has significant potential as a natural anticancer therapy option for breast cancer.

Conflict of Interest

The author`s declare no conflict of interest.

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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