The synergistic effects of 1,2-epoxy-3 (3-(3,4-dimethoxyphenyl)-4H-1-benzopiran-4on) propane and doxorubicin on breast cancer culture cell line

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Abstract. Breast cancer is the second leading cause of death due to the low success rate of cancer treatment. Breast cancer therapy has not been maximized that encourage researchers to look for effective and selective cancer drugs. The purpose of this study was to determine at the synergistic effects of 1,2-epoxy-3(3,4-dimethoxyphenyl)-4H-1-benzopiran-4on) propane (EPI) and doxorubicin on breast cancer cell cultures. This research is an experimental study in vitro, with a randomized study design post-test only control group design. The subject of this study was a T47D breast cancer cell culture, grown in a growth media with optimum time and incubation conditions. T47D are morphologically continuous cell lines like epithelial cells taken from breast tissue of a woman affected by ductal carcinoma. Cultures were performed using 30 wells, each well consisted of 1 x 10^6 cells. The results presented in the form of IC 50 using the Microculture Tetrazolium Salt (MTT) method and Combination index (CI) using compusyn software. An MTT method is a colorimetric assay based on cell metabolic activity. The results showed that the MTT method produced IC50 EPI values of 21.06μg/mL and doxorubicin 10.3μg/mL. EPI compounds in a combination of high concentrations have a CI below 1 (synergistic), whereas at high concentrations have CI above 1 (antagonistic). The conclusion of this study is that EPI compounds have weak anticancer in breast cancer and are synergistic with doxorubicin.

1. Introduction
Cancer is the highest cause of death in Indonesia. Among these cancers, breast cancer is the second leading cause of death after lung cancer, which is 12.9%. Breast cancer is cancer that ranks first for new cases in women, which is 43.3%. The high incidence of breast cancer is related to the increased risk factors for cancer, while the high mortality rate in breast cancer is due to the low success rate of cancer treatment. The low effectiveness of treatment is one of the factors that cause the low success of cancer therapy, which likely related to the occurrence of resistance, side effects, and toxicity. The ineffectiveness of treatment is also the cause of the high recurrence of cancer [1,2].
There are 4 cancer therapy modalities available, namely surgery, radiotherapy, chemotherapy, and hormones. Among these therapeutic options, chemotherapy is the main choice of therapy for advanced cancer. Doxorubicin is a chemotherapy drug widely used in cancer. The effectiveness of doxorubicin on cancer cells is very high, but the selectivity is still low, causing many side effects. The most common side effects of doxorubicin are cardiotoxic [3,4].

The ineffectiveness of cancer treatment, the high side effects and resistance, and the high costs required for cancer treatment underlie the emergence of studies to find anti-cancer drugs that have high effectiveness and selectivity [5]. To overcome this, one drug development that can be done is to use a drug combination. The drug combination is expected to improve the effectiveness and selectivity of treatment, reduce the side effects of drugs, prevent the occurrence of resistance and can reduce the dose of drugs used [6-8].

One of the targets for the development of anti-cancer drugs is the use of natural ingredients, especially medicinal plants. Active compounds that are known to have anti-cancer effects include flavonoids, which are secondary metabolites of plants. Flavonoids consist of several subclasses, one of which is isoflavones. A study shows that isoflavones in soy, namely genistein, can reduce the incidence of breast cancer. Genistein works as an antioxidant and antiproliferative, which can modulate gene expression in the cell cycle and apoptosis [9].

The use of soybeans as a source of producing genistein isolates turned out to require large amounts of soybeans and expensive costs, so it is necessary to look for other development alternatives to obtain compounds with genistein-like structures. Other compounds known to have genistein-like structures are found in clove leaf oil [10]. Clove leaves are found in Indonesia and until now their utilization has not been maximized.

The purpose of this study was to determine at the synergistic effects of 1,2-epoxy-3 (3-(3,4-dimethoxyphenyl)-4H-1-benzopiran-4one) propane and doxorubicin on breast cancer cell cultures.

2. Method

This research is in vitro experimental study, with a randomized posttest only control group design. The allocation of research samples was randomly selected.

The subject was a T47D breast cancer culture cell, grown in a growth media with optimum time and in an incubation conditions. T47D cell cultures performed using 30 wells, each consisted of 1 x 106 cells. T47D are morphologically continuous cell lines like epithelial cells taken from breast tissue of a woman affected by ductal carcinoma and has been established used for breast cancer research.

The research variables in this study are; concentration of 1,2-epoxy-3 (3-(3,4-dimethoxyphenyl)-4H-1-benzopiran-4one) propane and doxorubicin as the independent variables; Number of cell deaths, IC50 and Combination Index as the dependent variables and the number of T47D cells during planting, incubation time and temperature, CO2 content, and culture medium are the controlled variables.

2.1. Data collection technique

- IC50 is the concentration that can kill 50% of cancer cells, performed using Microculture Tetrazolium Salt (MTT) method. An MTT method is a colorimetric assay based on cell metabolic activity.
- Combination index (CI) measured, using Compusyn software
- The number of T47D cells calculated using the calculated chamber with the dilution method.

2.2. Analysis technique

The results will be presented in the form of IC 50 results (concentration that can cause the death of 50% of cancer cells, and CI (combination index) to show whether the compounds can be used as co-chemotherapy. The method used to evaluate drug combinations is the Combination Index (CI) with equation:
\[
CI = \frac{(D) \ 1}{(Dx) \ 1} + \frac{(D) \ 2}{(Dx) \ 2}
\]

Dx: The concentration of a single compound needed to give effect as much as a combination effect (D) 1, (D) 2: Concentrate the two compounds to give the same effect. The IC value is done with the Compusyn 1.4 software.

2.3. Interpretation method
The IC50 value of active compounds is determined by concentrations that can cause 50% cancer cells death.

IC50 is divided into 4 categories [11]:
- \( \leq 20 \ \mu g/mL \), active;
- \( >20 \text{–}100 \ \mu g/mL \), moderately active;
- \( >100 \text{–}1000 \ \mu g/mL \), weakly active; and
- \( >1000 \ \mu g/mL \), inactive

2.4. Conclusion technique
Table 1 showed the conclusion technique performed to assess whether the 1,2-epoxy-3 (3- (3,4-dimethoxyphenyl) -4H-1-benzopiran-4on compound) propane can be co-chemotherapy through the Combination Index (CI). Combination index is the standard measure for drug combination effect that indicates a synergistic effect (CI < 1), antagonistic effect (CI > 1) or additive effect (CI = 1) [6,12].

Table 1. Interpretation of CI value.

| CI value | Interpretation                      |
|----------|-------------------------------------|
| <0.1     | very strong synergistic effect      |
| 0.1 - 0.3| strong synergistic effect           |
| 0.3 - 0.7| synergistic effect                  |
| 0.7 - 0.9| mild synergistic effect - moderate  |
| 0.9 - 1.1| approaches the additive effect      |
| 1.1 - 1.45| mild - moderate antagonistic effects|
| 1.45 - 3.3| antagonistic effects                |
| > 3.3    | strong antagonist effects - very strong |

3. Results and discussion

![Figure 1. Bar graphic of IC 50 value.](image)

IC50 results, as can be seen in figure 1 showed that IC50 DOX value was lower than the compound (1,2-epoxy-3 (3- (3,4-dimethoxyphenyl) -4H-1 benzopiran-4on) propane. Index of the combination of
EPI and DOX compounds in a concentration of $\frac{1}{2}$ IC50 EPI and DOX is below 1 (very strong synergistic effect). This effect can be seen as a curve shown in figure 2 below.

![Figure 2](image)

**Figure 2.** Effect curve compounds (1,2-epoxy-3 (3- (3,4-dimethoxyphenyl) -4H-1 benzopiran-4on) propane and doxorubicin in T47D cells.

This study consists of two stages, stage I intended to determine the anticancer effect of (1,2 epoxy-3 (3- (3,4-dimethoxyphenyl) -4H-1 benzopiran-4on) propane (EPI) compounds on T47D breast cancer cell cultures. Stage 2 determine the co-chemotherapy effect of compounds (1,2 epoxy-3 (3- (3,4-dimethoxyphenyl) -4H-1 benzopiran-4on) propane (EPI) with doxorubicin (DOX), against T47D breast cancer cell cultures.

In the development of new anticancer drugs as candidates for cancer therapy agents, preclinical testing is one of the important things to understand the potential of cytotoxic activity. The cytotoxic test used as an initial screening to determine the effect of a natural substance in inhibiting tumor cell growth. A compound is considered to have anticancer properties if it is able to inhibit the growth of 50% population of tumor cells at a certain concentration. The conditions that must be fulfilled for the cytotoxicity test system include the ability of a testing system in reproducing dose-response curve with low variability, the number of cells and the information obtained from the dose-response curve must be in line with the appearance. One method commonly used to determine cell numbers is the MTT method [13,14].

In this study, cytotoxic test results of (1,2 epoxy-3 (3- (3,4-dimethoxyphenyl) -4H-1 benzopiran-4on) propane (EPI) compounds on T47D, using the method MTT produced IC50 EPI values in T47D cell culture of 21.06 μg / mL and IC50 of doxorubicin at T47D 10.3 μg / mL. With this IC50 value, it can be concluded that compounds (1,2 epoxy-3 (3- (3,4-dimethoxyphenyl) -4H-1 benzopiran-4on) propane are anticancer categories of moderately active compounds [11].

EPI compound is an isoflavone synthesized from clove leaf oil, its structure resembles genistein. Genistein is a multifunctional isoflavone, epidemiological studies show consumption of soy products containing abundant genistein can reduce the risk of cancer [15]. Previous research shows genistein can inhibit growth, cell survival, invasive, metastasis, and angiogenesis, while induce the apoptosis of various cancer cell, both in vitro and in vivo studies [16,17]. The activity of anticancer of genistein associated with its potency to trigger the apoptosis, it was mediated by activating Endoplasmic reticulum (ER) stress, increased the amounts of cleaved caspase-3 and cleaved PARP [18]. Excessive or prolonged stress can trigger apoptosis and cell damage, because ER is the main site for protein synthesis and modification, lipid biosynthesis and calcium storage, and plays an important role in maintaining cell homeostasis and survival [19].

The EPI structure consists of 3 double bonded rings, C2-C3 double bonds contribute to the conjugation between carbon and ring structures as anticancer candidates, through inducing cell death in various cancer cells such as colon adenocarcinoma and MDA-MB-231 breast cancer cells [20,21].
The problem faced in the application of chemotherapy is that chemotherapy agents are toxic to normal tissue, decreased immune system, and drug resistance occurs, for this problem it is necessary to apply co-chemotherapy agents or combination therapy. Combination therapy can increase the effectiveness of anticancer agents, use lower anticancer doses, decrease toxicity to normal tissue, slow down and inhibit drug resistance, and allow the efficacy of synergies in cancer cells. Combination testing with the isobologram method was carried out to determine whether the compound (1,2 epoxy-3 (3,4-dimethoxyphenyl) -4H-1-benzopiran-4on) propane could be a co-chemotherapy with potential doxorubicin [12,22].

The test results of a combination of compounds (1,2 epoxy-3 (3,4-dimethoxyphenyl) -4H-1-benzopiran-4on) propane (EPI) with doxorubicin (DOX) are presented in Table 1 and Figure 2. Index combination of EPI and DOX compounds in T47D cells in the ½ IC50 EPI and DOX constructs are below 1. Value shows that EPI compounds at that concentration synergize with DOX [5]. The best combination with the availability of 11% is obtained from a combination of ½ IC50 DOX compounds and ½ IC50 DOX.

Previous research showed isoflavones from clove leaf oil (1,2 epoxy-3 (3,4-dimethoxyphenyl)-4H-1-benzyopyran-4on) propane (EPI) induced cell death in HeLa cells and had a very synergistic co-chemotherapy effect with doxorubicin. Combinations of (1,2 epoxy-3(3,4-dimethoxyphenyl)-4H-1-benzyopyran-4on) propane with doxorubicin increased p53, TIMP-3, and miR-34a expressions in HeLa uterine cervix cancer cells [10]. The other studies have same result that genistein is synergic with some standard drugs in several cancer cell cultures such as pancreatic cancer, prostate cancer cell culture, lung cancer cells (H460), and breast cancer cells (MDA-MB-231) [23].

4. Conclusion

The conclusion of this study is that EPI compounds have moderately anticancer in breast cancer and are synergistic with doxorubicin. The results of this study can be used as a basis for further research relating to the mechanism of action of EPI in suppressing breast cancer cell growth and clinical trials in breast cancer patients.

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