

Original Article

Enhancing Anticancer Potential: Investigating the Synergistic Impact of Doxorubicin and Curcumin on HeLa and Vero Cells in Vitro

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Abstract: Cervical cancer ranks as the fourth most prevalent cancer globally and in Asia, standing as the second most common in Indonesia. Despite its efficacy, doxorubicin chemotherapy is associated with significant side effects. To mitigate these adverse effects, a promising approach involves combining conventional drugs with curcumin. Both curcumin and doxorubicin have demonstrated cytotoxic effects against cervical cancer (HeLa). This research aims to determine the synergistic effect of the combination of doxorubicin and curcumin on HeLa and its safety in normal cells (Vero cells). This study adopted an experimental design utilizing doxorubicin and curcumin samples with HeLa and Vero cells. The investigation was initiated with cytotoxic and combination tests using the MTT method. The obtained results included IC₅₀ values and combination indices, and the analysis involved a comparative examination of outcomes between HeLa and Vero cells. Cytotoxic tests revealed IC₅₀ values for doxorubicin and curcumin on HeLa cells, measured at 2.17 ± 0.06 and 26.37 ± 2.00 $\mu\text{g/mL}$, and 16.57 ± 5.56 and 172.22 ± 19.93 $\mu\text{g/mL}$ on Vero cells. Combination test results were represented by the combination index. The synergistic effect is observed in the combination of curcumin at a concentration of 9 $\mu\text{g/mL}$ and doxorubicin at a concentration of 0.125 $\mu\text{g/mL}$, resulting in a combination index of 0.50. These findings suggest a promising avenue for enhancing the therapeutic potential of doxorubicin in cervical cancer treatment while minimizing adverse effects.

Keywords: antiproliferative, cancer, cervical, combination, doxorubicin.

1. INTRODUCTION

The incidence rate of cervical cancer in Indonesia increased slightly from 7.4 to 8.7 per 100,000 women, while the prevalence rate increased from 43.3 to 52.4 per 100,000 women from 1990 to 2017 [1]. The incidence of cervical cancer in Indonesia is estimated to reach 180,000 new cases per year [2]. Cervical cancer is the number one killer in Indonesia with an incidence rate of 100/100,000 per year [3]. Due to this, many efforts have been made to reduce the incidence of cervical cancer.

Efforts made to reduce the incidence of cervical cancer are the important of providing strategic and evidence-based health services to reduce the impact of cervical cancer [4]. The incidence and prevalence of cervical cancer can be reduced through education, prevention, treatment, family planning, the use of regular pap smears, and appropriate management and follow-up after cases of cancer occur [5]. Reducing cancer cases can be done with surgery, radiation, and treatment using synthetic drugs, herbs, or combinations as co-chemotherapy.

Combination of synthetic drugs and traditional medicines as co-chemotherapy for cancer treatment. The results of co-chemotherapy research provide synergistic, additive, and antagonistic information. The effects on the results of combination research are shown by the combination index. The expected combination index price is in the range that shows a synergistic effect, namely below 1. Exploration of the potential synergistic effect of chemotherapy agents with active compounds of traditional medicines to increase anti-cancer properties, reduce side effects, increase penetration of chemotherapy agents into cancer cells, and provide supportive care to improve quality of life due to chemotherapy with anticancer drugs [6].

Anticancer drugs also have toxic effects on normal cells and low bioavailability in the body. Cancer drugs that are combined to reduce the incidence of cervical cancer include cisplatin, which is a drug of choice for cervical cancer, paclitaxel, and doxorubicin. Doxorubicin has toxicity in normal cells, so research into the addition of co-chemotherapy is needed [7]. The active substances used in co-chemotherapy include flavonoids, curcuminoids, and alkaloids.

The mechanism of action of curcumin can occur both internally (in mitochondria) and externally (via receptors). Cell surface transmembrane death). The intrinsic pathway typically begins with activation of the tumor suppressor p53, a cell cycle regulator, and via members of the B cell lymphoma (Bcl-2) family [8]. Upregulation of Bcl-2-activating p53 inactivates its homolog antagonist (Bak) and Bcl-2 x-linked protein (Bax), a pro-apoptotic member of the Bcl-2 family. Bak and Bax promote apoptosis by forming pores in the mitochondrial membrane that release cytochrome c into the cytoplasm and thereby activate caspases. Nuclear factor (erythroid derivative 2) like 2 (Nrf2) is a transcription factor involved in primary defense pathways against the effects of oxidative stress [9]. The Nrf2 pathway transcription factor is a regulator of genetic variations associated with the detoxification of electrophiles and ROS as well as the repair and elimination of damaged products caused by cancer cells. Curcumin has potential as a chemopreventive and antiproliferative agent that activates the Nrf2 pathway, restores p53, and modulates inflammatory molecules [8].

Curcumin is a group of curcuminoids that are abundant in turmeric (*Curcuma longa*). The potential of curcumin as co-chemotherapy is shown in the results of research in combination with cisplatin, which showed synergistic results as co-chemotherapy [8]. However, the response to this combination provides a different effect with a small impact on restraining the growth of cancer cells, and there are still side effects [10]. A combination with other chemotherapy needs to be done, namely doxorubicin. This is supported by research showing that *C. longa* extract can reduce the side effects of doxorubicin on breast cancer in vitro in 4T1 cells, where the content of *C. longa* is curcumin [11].

Co-chemotherapy testing of curcumin and the chemotherapy agent, namely doxorubicin, on normal cells, also needs to be carried out. This is related to the toxic effect of doxorubicin on normal cells and can cause side effects such as diarrhea, nausea, vomiting, and an increased risk of infection [12]. The parameter that indicates security in normal cells is the selectivity index. The selectivity

index value is obtained by comparing the IC₅₀ effect of the sample on cancer cells compared to the IC₅₀ of the sample on normal cells [13].

Curcumin shows promise as a co-chemotherapy for doxorubicin in breast cancer cells, however, the potential for co-chemotherapy in other cancer cells needs to be tested. Testing doxorubicin as a co-chemotherapy in cervical cancer cells is hypothesized to provide a synergistic effect because curcumin, like in breast cancer cells, can increase bioavailability. Testing curcumin as a doxorubicin co-chemotherapy in cervical cancer cells can provide important information regarding increasing the therapeutic potential of this combination. The aim of this study was to determine the increased effect of doxorubicin with curcumin co-chemotherapy and its safety on Vero cells.

2. MATERIALS AND METHODS

The research carried out was experimental research with the active substances curcumin and doxorubicin. The synergistic effect of anticancer activity was tested with the combination of these samples: HeLa cells (cancer cervix cells) and Vero cells (normal cells). The samples used in this study were curcumin and doxorubicin. In this study, MERCK-Schuchardt brand curcumin with a purity of $\geq 90\%$ was used. Curcumin and doxorubicin were obtained from the Phytochemical Laboratory of Ahmad Dahlan University. This research was conducted based on the ethical clearance issued by KEP UAD Number 012212203, 23 January 2023.

2.1. Cell culture and cytotoxic assay on HeLa and Vero cells

HeLa cells were grown in DMEM 1640 medium (Gibco) containing 10% volume fetal bovine serum (FBS) (Gibco) and 100 $\mu\text{g/ml}$ penicillin-streptomycin (Gibco) and incubated in a CO₂ incubator (Heraeus) at 37°C with flow 5% CO₂ [14]. Cytotoxicity test with the MTT test. HeLa cells were divided into 96-well plates (Nunc), a total of 5000 cells per well, and incubated with doxorubicin and curcumin either singly or in combination using DMSO solvent for 24 hours in a CO₂ incubator (Heraeus). The concentrations used for the cytotoxic test of doxorubicin in HeLa cells were 4.6; 2.3; 1.15 μM , and curcumin was 50, 25, and 12.5 μM . At the end of each HeLa cell incubation. 100 μl of MTT (Sigma) in DMEM medium (Gibco) was added to the wells. The plates were then incubated for 4 h at 37 °C until formazan crystals formed (viewed under an inverted microscope (Olympus CH-2)). After 4 hours, the MTT reaction was stopped by adding 10% SDS blocking reagent, 100 μl each, into the wells, then incubated overnight at room temperature covered with aluminum foil. The resulting violet color absorption intensity was read with an ELISA reader (Bio-Rad microplate reader reference sensor serial number 11565, Japan) at a wavelength of 595 nm to obtain the absorbance [14]. Next, the percentage of live cells was calculated, and then a linear regression of the relationship between cell concentrations and the percentage of viability cells was made. The same method is used for testing on Vero cells. The difference lies in the test concentration used. The concentrations of doxorubicin are 100, 25, and 3.125 $\mu\text{g/mL}$ and the concentrations of curcumin are 50, 25, and 12.5 $\mu\text{g/mL}$.

$$\text{Percentage of viability cells} = \frac{(\text{sample absorbance} - \text{media control absorbance})}{(\text{solvent control absorbance} - \text{media control absorbance})} \times 100\% \dots\dots\dots(1)$$

2.2. Test the selectivity

The cell selectivity test was carried out as in the cytotoxic test, with doxorubicin and curcumin samples tested on HeLa cells and Vero cells. The selectivity index is determined by comparing the IC₅₀ of the test material on Vero cells to the IC₅₀ of the test material on HeLa cells [15].

2.3. Test the combination

The samples used were doxorubicin and curcumin, as well as doxorubicin and curcumin combination. Testing is carried out on the IC₅₀ results of the samples, which are then combined at the IC₅₀ concentration of the single sample. Absorbance is used to calculate living cells under the influence of a single sample, which is then included in the Combination Index calculation formula (Equation 2). Dx is the IC₅₀ concentration of a single compound, and D1 and D2 are the combination concentrations that provide an effect equivalent to a single concentration [16].

$$CI = \frac{[D]1}{(Dx)1} + \frac{[D]2}{(Dx)2} \dots\dots\dots(2)$$

3. RESULTS AND DISCUSSION

The results obtained from this study were the combination index of doxorubicin and curcumin. Cytotoxic tests for single compounds are carried out first to design the combination tests to be carried out. The results of the single compound cytotoxic test are in the form of IC₅₀ which is then used as the basis for the concentration used for the combination test of doxorubicin and curcumin.

3.1. Cell culture and compound cytotoxicity test on HeLa and Vero cells

HeLa and Vero cell cultures were carried out to obtain IC₅₀ and see safety by comparing Vero cells and HeLa cells. Figure 1 shows a picture of HeLa cells in controls that appear to be in greater numbers compared to the effect of doxorubicin and curcumin. Figure 1 displays the findings of the log concentration vs percent viability cells relationship. This is in line with the IC₅₀ results obtained on HeLa cells shown in Table 1. The IC₅₀ results on Vero cells show that doxorubicin to normal cells=16.57±5.56 and curcumin=172.22±19,93, IC₅₀ on HeLa cells doxorubicin=2.17±0.06 and curcumin = 26.37±2.01 µg/mL. The results of Vero and HeLa cells affected by doxorubicin and curcumin are shown in Figure 2.

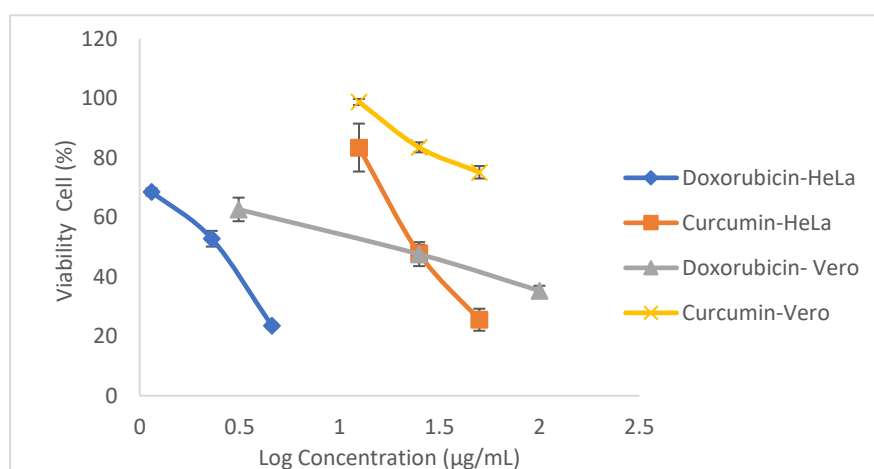


Figure 1. Correlation between doxorubicin and curcumin log concentrations vs HeLa and Vero Cell viability(%)

Doxorubicin has been shown to intercalate deoxyribonucleic acid (DNA) and bind to and subsequently inhibit DNA polymerase, both of which lead to an inhibition of DNA synthesis. It works by damaging the DNA inside the cell, which leads to cell death [17]. However, it can also cause significant side effects, such as nausea, vomiting, hair loss, an increased risk of infection, and bleeding. The optimal dose and duration of doxorubicin treatment depend on the type and stage of the cancer being treated. Based on this, this research was continued with selectivity tests on normal cells [18].

Figure 2's results demonstrate that the addition of doxorubicin does not significantly alter the image of normal cells, but it does alter the image of HeLa cells, which have fewer images. This is in line with research showing that doxorubicin has a selectivity index on cervical cells [19].

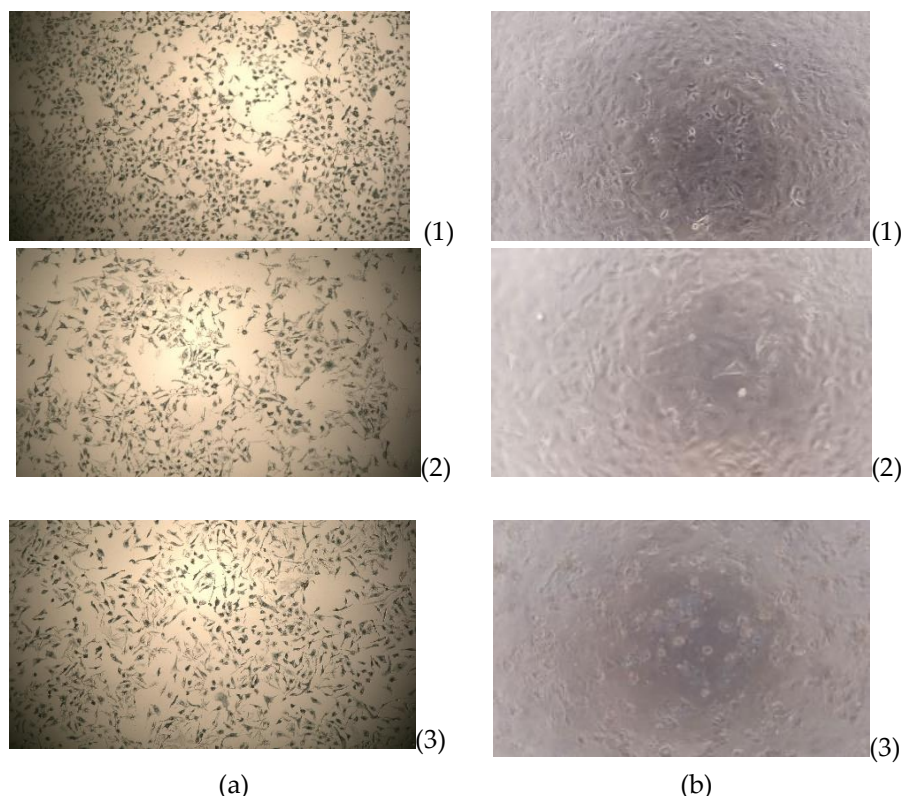


Figure 2. Image of Vero cells and HeLa cells (a) Vero cells, (b) HeLa cells, and (1) control cells; (2) doxorubicin, and (3) curcumin

3.2. Test the selectivity

The selectivity index (SI) of curcumin and doxorubicin against HeLa cells was calculated using the selectivity index parameter. The selectivity index is calculated by comparing the IC₅₀ of Vero cells against HeLa cells. The selectivity index results for doxorubicin and curcumin were 7.64 and 6.53, respectively. This shows that doxorubicin and curcumin are selective in HeLa cells.

Tabel 1. IC₅₀ and Selectivity Index doxorubicin and curcumin to vero and HeLa cells

Compound	IC ₅₀ (µg/mL)		Selectivity Index
	Vero	HeLa	
Doxorubicin	16.57±5.56	2.17±0.06	7.64
Curcumin	172.22±19.93	26.37±2.01	6.53

A high SI value (>2) of a compound gives the selective toxicity against cancer cells ($SI = IC_{50}$ normal cell/ IC_{50} cancer cell) [20]. The selectivity index for vero and HeLa cells for the compound doxorubicin was 7.64 and curcumin was 6.53. Based on these results, it is necessary to carry out tests related to the combination of the two to reduce the side effects of doxorubicin by reducing its concentration.

3.2. Test the combination

After knowing the IC_{50} of curcumin and doxorubicin against HeLa cells, a combination cytotoxic test was continued based on multiples of the IC_{50} results obtained previously. This is done to reduce the risks of using a combination of curcumin and doxorubicin. The combination series used for curcumin are 9; 4.5; 1, 2.5; and 1.25 $\mu\text{g/mL}$. Meanwhile, the concentration series used for doxorubicin are 1; 0.5; 0.25, and 0.125 $\mu\text{g/mL}$ as seen in Table II.

Table 2. Combination Index of Curcumin and Doxorubicin in HeLa cells

Concentration of Doxorubicin ($\mu\text{g/mL}$)	Concentration of Curcumin ($\mu\text{g/mL}$)	Combination Index
1	9	2.20
0.5	9	1.27
0.25	9	0.93
0.125	9	0.50
1	4.5	2.49
0.5	4.5	3.32
0.25	4.5	3.39
0.125	4.5	6.31
1	2.5	2.04
0.5	2.5	3.41
0.25	2.5	3.10
0.125	2.5	1.44
1	1.25	2.02
0.5	1.25	3.60
0.25	1.25	1.96
0.125	1.25	3.17

The results of calculating the combination index values obtained from 16 combinations of curcumin and doxorubicin against HeLa cells at a concentration of 9 $\mu\text{g/mL}$ curcumin and 0.25 $\mu\text{g/mL}$ doxorubicin are 0.93 and a concentration of curcumin of 9 $\mu\text{g/mL}$ and doxorubicin of 0.125 $\mu\text{g/mL}$ with a combination index value of 0.50. The interpretation of the combination index value is that curcumin and doxorubicin have a mild-moderate synergistic effect and have a synergistic effect on HeLa cells on the combination curcumin of 9 $\mu\text{g/mL}$ and doxorubicin of 0.125 $\mu\text{g/mL}$.

The synergistic effect of the combination of curcumin of 9 $\mu\text{g/mL}$ and doxorubicin of 0.125 $\mu\text{g/mL}$ is shown by the combination index based on previous research, which states that when the combination index is <1 , then the combination is in the synergistic effect range [21]. This is in line with research that states that the combination of curcumin and doxorubicin showed a synergistic

effect in enhancing the anticancer activity in gastric adenocarcinoma cells [22]. Curcumin is used as a co-chemotherapy against doxorubicin because curcumin can increase the anticancer effect of doxorubicin, possibly by increasing the absorption of doxorubicin. Apart from that, curcumin also protects normal cells that are damaged by doxorubicin through the mechanism of reducing oxidative stress, which can damage DNA. Thus, curcumin has the potential to act as a co-chemotherapy for doxorubicin in reducing the toxic effects [22]. Curcumin has been known to reduce the adverse effects of DOX on normal cells and tissues by reducing inflammation, oxidative stress, and apoptosis. The current development related to the development of curcumin as a co-chemotherapy is the development of nanoparticles [23]. The potential of curcumin as co-chemotherapy needs to be developed, considering that chemotherapy has toxicity to normal cells.

4. CONCLUSION

The combination of doxorubicin at a concentration of 0.125 µg/mL and curcumin at a concentration of 9 µg/mL produces a synergistic effect with a combination index of 0.50.

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