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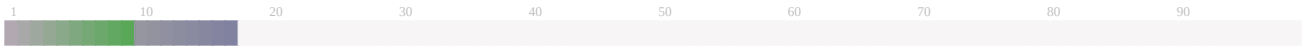

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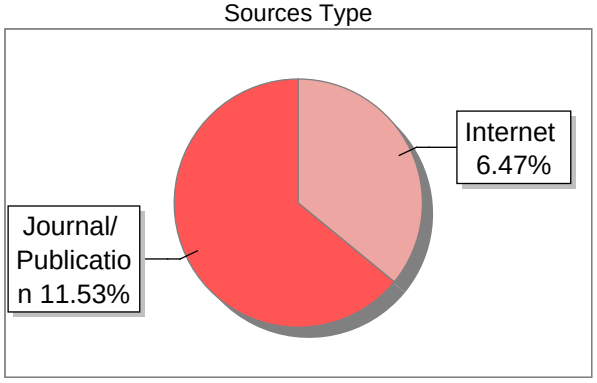

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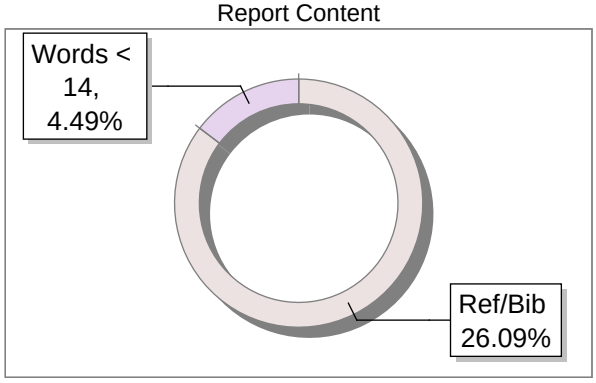

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Original Research Article

Co-Chemotherapy Effect of The Extract of *Hibiscus Sabdariffa* and Cisplatin Against Apoptosis and Anti-Proliferation on T47d and Vero Cells

Citra Ariani Edityaningrum[1], Amanda Khairurrizki[1], Laela Hayu Nurani[*1], Moch Saiful Bachri[1], Sapto Yuliani, Dwi Utami[1], Kintoko[1], Nurkhasanah[1], Lalu Muhammad Irham[1], Zainul Amiruddin Zakaria[2]

[1]*Faculty of Pharmacy, Universitas Ahmad Dahlan, Prof. Dr. Soepomo SH street, Yogyakarta, Indonesia, 55164*
[2]*Faculty of Medicine and Health Science University Sabah Malaysia, Kinabalu, Sabah, Malaysia, 88400*

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ABSTRACT

The use of cisplatin as a breast cancer chemotherapy agent has side effects, one of which is toxicity to normal cells. Therefore, this study aimed to use *Hibiscus sabdariffa* extract (HSE) to promote cytotoxic prevention. Several active compounds such as anthocyanin, protocatechuic acid, and hydroxyl citric acid compounds in HSE have cytotoxic activities. Combining cisplatin and the extract of *Hibiscus sabdariffa* (HSE) as a co-chemotherapy agent reduces the dose of cisplatin. It was discovered that HSE and cisplatin had cytotoxic effects on T47D cells with IC_{50} values of 32.3 ± 2.15 µg/mL and 6.64 ± 0.68 µg/mL, respectively. Selectivity assay on Vero cells resulted in SI values of 52.84 for HSE and 1220.7 for cisplatin. Anti-proliferation test on control cells indicated a doubling time value of 55.74 ± 0.55 h, while HSE at concentrations of 32.3 µg/mL,16.15 µg/mL, and 8.08 µg/mL didn't indicate any doubling time value. Based on this study, we found that the combination of 16.15 µg/mL of HSE and 3.32 µg/mL of cisplatin had a strong synergistic effect with a Combination Index (CI) value of 0.002. The mechanism of HSE and cisplatin was identified through the increasing expression of p53 and decreasing expression of Bcl-2.

Keywords: Apoptosis activity, cisplatin, *Hibiscus sabdariffa,* co-chemotherapy, cytotoxic effects

Introduction

Globally, breast cancer is the leading cause of mortality[1]. Genetic and hormonal endogenous factors are the primary causes of breast cancer. Endogenous genetic factors are attributable to gene mutations and inherited genes that carry the characteristics of breast cancer. Meanwhile, endogenous hormonal factors are spurred by the increase of estrogen hormones in the body, both relatively and absolutely[12]. One of the most common changes in expressions and functions in breast cancer is brought about by the presence of gene mutation on p53[3]. The role of p53 includes its potency to induce apoptosis and inhibit cell cycles. Mutation of p53 causes its protein expression to decrease, which can reduce the apoptosis ability and allow cells to continue to grow into cancer cells[4].

The molecular mechanism of apoptosis consists of three stages of cancer cell inhibitions, namely initiation by apoptosis-inducing compounds, activation of caspases, and release of cellular components (proteolysis)[5]. Caspases (cysteine proteases with aspartate specificity) play an important role in both apoptosis regulation and decimation. There are two groups of caspases, namely initiators (caspases 8, 9, and 10) and effectors (caspases 3, 6, and 7). The protein families Bcl-2, p53, Apaf, and caspases play an active role in regulating apoptosis.

*Corresponding author. E mail: laela.farmasi@pharm.uad.ac.id
Tel: +62 898-3464-706

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The Bcl-2 protein family includes anti-apoptotic proteins (Bcl-2 and Bcl-xL), pro-apoptotic proteins (Bax, Bak, and Bad), and BH3 proteins (Bid, Bim, Bik, and PUMA/p53 up-regulated modulator of apoptosis). Anti-apoptotic proteins regulate apoptosis by blocking the release of mitochondrial cytochromes, while pro-apoptotic proteins act by promoting the release[6]. The variations in apoptosis mechanisms are affected by protein expression that occurs in cancer cells[7].

The chemotherapy agents used in breast cancer treatment are cisplatin, doxorubicin, fluorouracil, and carboplatin[8]. However, the use of chemotherapy agents has toxic effects on normal tissues. To reduce the toxicity effects on normal tissues, combination therapy with a co-chemotherapy agent is needed[9]. Anticancer and co-chemotherapy compounds as alternative treatments can be extracted from *Hibiscus sabdariffa* Linn. Extracts of *Hibiscus sabdariffa* (HSE) also protect the hepatotoxic problem of mice-induced paracetamol[10]. This herb contains alkaloids, saponins, terpenoids, anthocyanins, flavonols, quercetins, polyphenols, and various essential components that are commonly associated with antioxidant and anticancer activities. Initial experiments discovered cytotoxic, anti-proliferative, anti-mutagenic, apoptotic, anti-angiogenic, and anti-metastatic activities in HSE[11,12]. The flavonoids isolated from HSE enable the regulation of several signaling pathways, including Bcl-2 modulation through the p53 pathway which targets apoptosis, inflammation, and oxidative stress, resulting in a potential anticancer effect[13].

A previous study reported that HSE is a potential source of anticancer according to the result of a cytotoxic test on T47D cells using the MTT method, with an IC_{50} value of 55.7 ± 3.17 µg/mL[14]. *H. sabdariffa* Linn's methanol extract has limited anticancer activity against the breast cancer cell MCF-7 (IC_{50} value: 112.1 µg/mL)[15]. Treatment using roselle (20 µg/mL) increases the cytotoxic activity of cisplatin both in A549 and H460 cell lines. Previous studies also found that the combination of roselle extract and cisplatin effectively

increased the efficacy of cisplatin in cancer treatment by promoting apoptosis[16]. However, the combination of HSE as a co-chemotherapy agent and cisplatin to obtain a synergistic effect on breast cancer, particularly on T47D cells, has never been studied previously.
Therefore, a study concerned with combining HSE and cisplatin to reduce toxic effects on normal cells should be encouraged. A previous study reported the efficacy of HSE as an anti-cancer on one sample. However, the effectiveness of HSE as a co-chemotherapy agent to enhance cisplatin's efficacy in increasing apoptosis and anti-proliferative effect on breast cancer cells, particularly T47D, has not been evaluated. The findings of this study are projected to demonstrate the importance of employing HSE as an adjunct chemotherapy agent in the treatment of breast cancer.

Materials and Methods

Plant Collection and Identification
HSE simplicia was obtained from CV Herbal Anugrah Alam (Banguntapan, Salakan, Bantul, Yogyakarta, Indonesia, 7°45'37.4"S 110°24'31.2"E) with a statement of material legality, certificate number 01/009/HAA/IV/2023. *Hibiscus sabdariffa* was identified by a biologist from Universitas Ahmad Dahlan, Drs. Hadi Sasongko, M.Si. T47D and Vero cells were utilized as test subjects; they were taken from *In Vitro* Cell Culture Laboratory, Parasitology Laboratory, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University. T47D cells were kept in M119 medium, supplemented with FBS, and cultured by flowing CO_2 at a 5% concentration at a temperature of 37°C[17].

Hibiscus sabdariffa Extraction
The extraction process of *Hibiscus sabdariffa* was carried out using the maceration method. Dried HS simplicia (7.5 kg) was extracted by maceration with 17.8 L of 96% ethanol solvent. The maceration process was conducted for 24 h, with stirring for an hour. The solution was filtered, resulting in filtrate and precipitate. The filtrate was freeze-dried with a freeze-dryer (Leybold, Swedish), concentrated with a rotary evaporator (Heidolph, Germany), and evaporated with a water bath at 50°C until a condensed extract was obtained. The precipitate was re-macerated with the same solvent up to two times[18].

Cytotoxic Test, Selectivity Test, and Anti-proliferation Test on T47D cells
Tests were carried out with ethical clearance by KEP UAD, dated August 4, 2023, number 0123071177. Cells were placed on a 96-well plate (each well contained 2 x 10^4 cells). Incubation was performed overnight with a liquid CO_2 concentration of 5% at a temperature of 37°C (model: Incubator CO_2: Hera cell, United States). Different extract concentrations (15.625–1000 µg/mL) were added to each well, followed by incubation for 24 h. The following step involved filling each well with 10 µL of 0.5% MTT in PBS, followed by incubation for an additional 24 h. After an addition of 10% SDS, the process was halted and kept going for 4 h. The purple color that resulted was then measured at a wavelength of 595 nm using an ELISA reader (Biorad, England)[19]. The following formula was used to convert the absorbance values, which were the forms taken by the collected data, to percentages of living cells (Equation 1).

$$Percentage\ of\ living\ cells = \frac{(sample\ absorbation - medium\ absorbation)}{(control\ absorbation - medium\ absorbation)}\ x\ 100\% \text{.....(eq. 1)}$$

The sample concentration and cell viability values obtained were included in a linear regression equation to obtain the IC_{50} value:
y = bx + a……(eq. 2).

Based on the IC_{50} values from a single cytotoxicity test, a concentration series consisting of 1/2 IC_{50}, 1/4 IC_{50}, and 1/8 IC_{50} for HSE and cisplatin was performed[20]. Anti-proliferation test was carried out by using a similar test as the cytotoxic test, involving incubation for 0, 24, 48, and 72 h. Doubling time as an anti-proliferation test parameter was obtained from a graph of the relation between incubation time (hours) and cell viability using the following equation:

$$Doubling\ time = \frac{Y - A}{B}\ x\ 100\% \text{ ..... (eq. 3)}$$

Y = log (2 x initial number of living cells)
A = intersect
B = slope
The selectivity of EBR against Vero cells and T47D cells was determined based on the IC_{50} obtained from the cytotoxic test. The selectivity parameter is expressed by the Selectivity Index, which is determined by comparing IC_{50} on Vero cells and IC_{50} on T47D cells. The extract is said to have high selectivity if the SI value is > 3, and it is said to be less selective if the SI value is < 3[21].

Combination Index Test
To test the combination index, 100 µL of cells were distributed into 96-well plates and incubated for 24 h so they could adapt and attach to the wells. Then, 50 µL of each concentration, i.e., IC_{50}, 1/2 IC_{50}, ¼ IC_{50}, and 1/8 IC_{50}, of HSE and cisplatin was added to each well and incubated for another 24 h. Subsequently, 50 µL of MTT reagent was added to each well, including the media control (without cells), and incubated for 4 h or until formazan was formed, added 10% SDS stopper which was dissolved in 0.1 N HCl. The absorbance values were observed using an ELISA reader at a wavelength of 595 nm[22].

Immunocytochemistry Test
Immunocytochemistry assay was performed by adding 1000 µL of sample in RPMI 1640 culture medium to the cell suspension in each well (with a cell density of 5 x 10^5 cells/mL) until the final concentration range in the well was reached, specifically 24.45 µg/mL. In the control group, 1000 µL of the sample was replaced with 1000 µL of culture medium. The plate was incubated with 5% CO_2 for 24 h at a temperature of 37°C. After that, the cells were taken out and smeared onto a poly-l-lysine slide. The immunocytochemistry test was conducted using primary monoclonal antibodies (IgG) against p53, Bax, caspase-3, and Bcl-2, and the results were examined under a light microscope. Brown/dark color was produced by cells that expressed the proteins p53, Bax, caspase-3, and Bcl-2. Purple/blue color was produced by cells that did otherwise. The number of cells was counted in a certain area for both results[23].

Data Analysis
Cytotoxic Test, Selectivity Test, and Anti-proliferation Test
The percentage of living cells was determined using Equation 1. The selectivity index on Vero cells was calculated using Equation 4.

$$SI = \frac{IC50\ on\ Vero\ cells}{IC50\ on\ T47D\ cells} \text{ ....(eq. 4)}$$

HSE and Cisplatin Combination Test
The Combination Index value was calculated using the Compusyn application. Combination Index values were yielded for all treatments.

Immunocytochemistry Test
Antibody expression analysis was carried out by comparing the expression of antibodies in cells given the samples and in those that were not. The percentage of expression was calculated using Equation 5.

$$Percentage\ of\ expression = \frac{The\ number\ of\ cells\ expressing\ antibodies}{The\ number\ of\ living\ cells}\ 100\% \text{..(eq. 5)}$$

Results and Discussion

The HSE maceration process produced a yield of 15.87% (7.52 kg). TLC conducted in previous research revealed that HSE is positive for flavonoid and tannin compounds[24].Cytotoxic assay was conducted to determine the cytotoxic effect of HSE and cisplatin on T47D and Vero cells. The cytotoxic test served as an initial step to determining the concentrations of compounds used in the subsequent tests, including the Combination Index test. IC_{50} (median inhibitory concentration) was used as a parameter representing cytotoxic effect[25].
Based on the IC_{50} value, the antitumor impact was categorized into four groups according to the modified NCI (National Cancer Institute)

criteria Srisawat et al. [26]: IC_{50} ≤2 0 µg/mL indicates active toxicity, IC_{50} 20–100 ug/mL indicates moderately active toxicity, IC_{50} 100–1000 µg/mL indicates barely active toxicity, and IC_{50} > 1000 µg/mL indicates inactive toxicity[23]. Additionally, the NCI IC_{50} value categories demonstrates that a compound's potential toxicity decreases with increasing IC_{50} value. Cytotoxic test was carried out on both cancer and normal cells to determine the toxicity of certain compounds on normal cells. The results of the test are presented in Table 1 and Figure 1.

With an IC_{50} value of 32.3 ± 2.15 µg/mL, greater than the IC_{50} value of cisplatin (6.64 ± 0.68 µg/mL), HSE was classified as very active. The fact that cisplatin is a chemotherapy agent that has been tested and used explains its greater sensitivity to cancer cells. The result of the study is consistent with that of a previous study, which classified HSE as active with an IC_{50} value of 55.7 ± 3.17 µg/mL[14].

The cytotoxic effect on cells stems from the compounds contained in HSE, namely anthocyanins, flavonoids, quercetins, polyphenols, and saponins. Anthocyanins can modulate the activity of various targets in carcinogenesis through direct interactions or by modulating gene expression, causing inhibited cell growth[27]. The antioxidant effects of anthocyanins can be observed in *in vitro* research using cultured breast, ovarian, colon, endothelial liver, and leukemia cancer cells. This effect takes the form of cell proliferation inhibition and is anti-carcinogenic activities[28]. Anthocyanins induce inhibition of cell proliferation by blocking the cell cycle at different stages. Besides, they can induce apoptosis in cancer cells by internal and external mitochondrial pathways. They can also inhibit invasion and suppress angiogenesis and metastasis of cancer cells[29]. As for quercetins, although they have very little effect on normal cells they can cause considerable toxicity in breast cancer cells. During the progression of the cell cycle, quercetins cause S phase arrest, which is followed by apoptosis[30].

Since the IC_{50} values of HSE and cisplatin against Vero cells were 1.707 ± 1.321 µg/mL and 8.160 ± 1.203 µg/mL, respectively, the SI values for HSE and cisplatin were, respectively, 52.84 (> 3) and 1220.7 (> 3). These results showed that HSE and cisplatin act selectively on normal cells. Selectivity assessment was conducted by comparing IC_{50} on normal cells and IC_{50} on cancer cells. A sample would be classified as selective if the SI (Selectivity Index) value was greater than or equal to 3, and SI values lower than 3 would indicate non-selectivity[19].

Inhibition of Cell Proliferation Test

A test of cell proliferation inhibition was carried out using the MTT method. The purpose of the assay was to observe the effect of HSE on the doubling time of T47D cells. Doubling time is the time it takes for cells to grow twice as large[31]. Compounds that delay the cells' doubling time may inhibit genes or proteins that regulate the cell cycle[32]. The concentrations of HSE used in the test were IC_{50}, ½ IC_{50}, and ¼ IC_{50}.

According to Table 2, the control cells had a doubling time value of 55.74 ± 0.55 h when the log of number of living cells was linearly regressed against time. This resulted in a slope value of 4.7 x 10^{-4}. Regression of the sample at concentrations of 32.3 µg/mL, 16.15 µg/mL, and 8.07 µg/mL resulted in negative slopes and showed no doubling time because of cell death. Based on these results, it can be concluded that HSE at these concentrations has anti-proliferative activity on T47D cells.

Table 1: Cytotoxicity test of Hibiscus sabdariffa extract and cisplatin on T47D and Vero cells

Treatment	IC_{50} (µg/mL)	
	T47D	Vero
HSE	32.3 ± 2.15	1707 ± 1.321
Cisplatin	6.64 ± 0.68	8106 ± 1.203

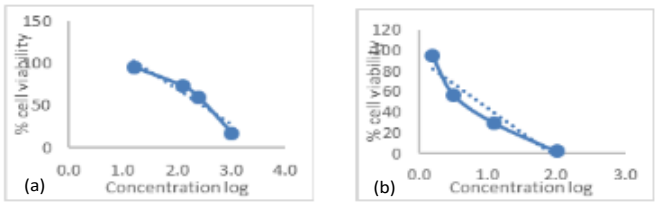


Figure 1: Cytotoxic effect of (a) Hibiscus sabdariffa extract and (b) cisplatin

Combination Index Test

The concentration variations used for the combination cytotoxic test were based on multiples of the IC_{50} results obtained previously. Value interpretation of HSE and cisplatin on T47D is shown in Table 3. The results of the cytotoxic tests showed that the combination of HSE and cisplatin produced various values of the combination index, ranging from synergistic to antagonistic effects. The combination of HSE and cisplatin exhibited a strong synergistic effect, as a Combination Index (CI) value below 0.9 shows such an effect, with the greatest CI value obtained being 0.002. The strong synergistic effect was obtained with HSE and cisplatin concentration of 15.16 µg/mL and 3.32 µg/mL, respectively. The results indicated that the cytotoxic effect of the HSE and cisplatin combination is greater than the sum of each single-dose compound effect. These results are credited to the phytochemical compounds contained in HSE, namely flavonoids and saponins.

Cisplatin and flavonoid combination induces depletion of *cellular* oxidative machinery, helps mitochondria to dysfunction, and causes apoptosis[33,34]. Flavonoids have been demonstrated to considerably boost the efficacy of cisplatin treatment when compared to cisplatin given as a single dose for the treatment of gastric cancer[35]. A biological mechanism exists in the cisplatin and HSE combination that guards against nephrotoxicity resulting from p53, multigene-activated protein kinase (MAPK), and protein kinase B (AKT) insufficiency to prevent apoptosis[35]. Another study found that saponins can protect against nephrotoxicity induced by cisplatin. Saponins cause cell resistance because cisplatin becomes sensitized by inducing apoptosis in ovarian cancer[36].

The synergistic increase in cytotoxic effect that occurs in the combination of HSE and cisplatin needs to be investigated further. This increase in cytotoxic effect can occur due to the apoptosis stimulation in T47D cells. Therefore, to observe if there were any indications of accelerated apoptosis, analysis was carried out using immunohistochemistry methods on the proteins involved in the apoptosis mechanism.

Apoptosis Mechanism Test with Immunocytochemistry

Observations of apoptosis regulatory protein expressions using immunocytochemistry methods adhered to the principle of binding specific antibodies. The binding of apoptotic proteins is indicated by the cytoplasm turning brown. The test results in this study are shown in Table 4. Each treatment showed a different increase in the expression of p53 and Bcl-2, suggesting that each treatment has a different response to apoptosis under single or combined conditions. This is due to the chemical contents of HSE, namely polyphenols, flavonoids, anthocyanin, and tannins[37].

Table 2: Doubling time of T47D cells with Hibiscus sabdariffa extract

Treatment	Equality		R	Doubling time
	Intercept	Slope		
HSE 32.3 µg/mL	3.844	-0.009	0.928	-
HSE 16.15 µg/mL	4.037	-0.017	0.937	-
HSE 8.08 µg/mL	4.043	-0.019	0.941	-
Control cells	3.471	0.0047	0.9524	55.74 ± 0.55

Table 3: Hibiscus sabdariffa extract and cisplatin Combination Index values (T47D cells)

	Cisplatin (µg/mL)				
HSE		6.64	3.32	1.66	0.83
(µg/mL)	32.3	0.051	0.021	0.016	0.014
	16.15	0.054	0.002	1.89	3.77
	8.07	0.332	3.033	10.678	12.195
	4.03	0.276	1.292	39.28	41.515

Reactive oxygen species (ROS) such as superoxide (O_2^-), singlet oxygen ($^1O_2$), peroxiyl radicals (ROO•), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•) can be eliminated thanks to the antioxidant effect of anthocyanins. The *in vitro* antioxidant activity of anthocyanins has been shown to have antitoxic and antitumor effects in several cell culture systems, including colon, breast, liver, endothelial, and leukemia cells. Anthocyanins induce apoptosis in both the intrinsic (mitochondria) and extrinsic (FAS) pathways. In the intrinsic pathway anthocyanins can cause increased mitochondrial membrane potential, increased release of cytochrome c, and caspase-dependent modulation of anti- and pro-apoptotic proteins. In the extrinsic pathway, anthocyanins modulate the expression of FAS and FASL (FAS ligand) in tumor cells and cause cell death[38]. In addition, the extrinsic pathway can induce death ligands to bind to death receptors, thereby modulating procaspase-8 which then forms the death-inducing signaling complex (DISC). This complex causes caspase-8 to be activated, which is then able to activate caspase-3 by cutting certain segments of procaspase-3. Activation of caspase-3 can also be performed in the intrinsic pathway involving caspase-9. Meanwhile, activation of p53 by several factors, oxidative stress, for example, will modulate the release of pro-apoptotic proteins in mitochondria, such as Bax and BAK. These proteins will stimulate the release of cytochrome c from the mitochondrial intermembrane and then activate Apaf-1, which forms a heptameric complex. The complex modulates and activates procaspase-9, and then caspase-9 will activate caspase-3[39]. It has been reported that anthocyanins in breast cancer cells can mediate apoptosis by affecting morphological changes and induce apoptosis by increasing caspase-3[29].

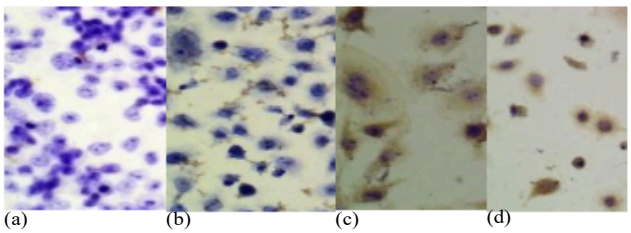
The quercetin compound can reduce Bcl-2 expression significantly and increase Bax expression significantly ($p < 0.05$). Therefore, it can be concluded that quercetins can inhibit cell growth and induce apoptosis in breast cancer cells[40]. The Bcl-2 gene is a protein that regulates the release of cytochrome c (cyto-c) from mitochondria, one of the stages in the process of cell apoptosis. Bcl-2 will inhibit the release of cyto-c from mitochondria to the cytosol. As a result, cyto-c will not be combined with Apaf-1, and no apoptosome will be formed, resulting in no apoptosis. Therefore, overexpression of Bcl-2 can cause cells to be resistant to apoptosis. The presence of smaller inhibition in the combination indicates that there is activity to inhibit cancer because Bcl-2 is a gene that plays a role in apoptosis[41]. Combination therapy (co-chemotherapy) aims to increase the effectiveness of treatment and reduce the side effects of chemotherapy agents. This study shows that the combination of roselle flower petal extract and cisplatin can stimulate the expression of p53 and can inhibit Bcl-2.

Conclusion

The use of HSE as a co-chemotherapy agent to reduce the side effects of cisplatin has a synergistic effect on T47D cells, with a combination index value of 0.0002 µg/mL. Doubling time of treatment groups at a concentrations of 32.3 µg/mL, 15.16 µg/mL, and 8.08 µg/mL yielded negative slopes. The absence of an indication of doubling time proved that HSE has anti-proliferative activity. On the other hand, the control cells showed an indication of doubling time of 55.74 h. The combination of HSE and cisplatin was found to be able to stimulate apoptosis by inducing gene expression of p53 and inhibiting Bcl-2 on T47D (Figure 2), with percentages of expression of 80.1% and 14.35%, respectively. These study results suggest a considerable prospect for using HSE as a co-chemotherapy agent to reduce the apoptosis effect of cisplatin during cancer therapy.

Table 2: p-53 and Bcl-2 expression in T47D cells

Treatment	% Gene expression	
	Caspase-3	Bcl-2
Cisplatin	10.09 ± 6.20	41.43 ± 5.80
HSE	9.36 ± 2.96	29.01 ± 7.38
HSE + Cisplatin	20.25 ± 3.52	14.36 ± 3.60



(a) (b) (c) (d)

Figure 2: Microscopic results of gene expression of (a) p53 (control), (b) p53 (combination of Hibiscus sabdariffa extract + cisplatin), (c) Bcl-2 (control), and (d) Bcl-2 (combination of Hibiscus sabdariffa extract + cisplatin)

Conflict of Interest

The authors 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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