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Cytotoxic Potential of Combination of *Hibiscus sabdariffa* L. Extract and Cisplatin against HeLa Cervical Cancer Cell Line: A Study of Antiproliferative Activity and Apoptosis Induction

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ABSTRACT

Cervical cancer is one of the most prevalent cancer types, making the development of effective anticancer agents critical. Cisplatin (CIS) is a drug that has been used to treat cancer, but it also affects normal cells. *Hibiscus sabdariffa* L. extract (HSE), which has the potential as an anticancer agent, can be developed as a co-chemotherapy with CIS. This study aimed to determine the potential of HSE as a co-chemotherapy with CIS against HeLa cervical cancer cells and determine specific and non-specific parameters of the studied extract. *Hibiscus sabdariffa* L. (HS) simplicia powder was macerated with 96% ethanol. An *in vitro* evaluation was carried out on antiproliferative activity by calculating the doubling time. Additionally, a selectivity test was conducted to calculate combination index (CI) values based on the microtetrazolium (MTT) method. Apoptosis mechanisms were explored based on immunocytochemical methods using p53, caspase, and Bax antibodies, followed by an observation of apoptotic induction using a flow cytometer. The HSE and CIS selectivity index values obtained for Vero cells and HeLa cells were 209 and 278, respectively. The antiproliferation test results showed that the combination of HSE and CIS could better extend the doubling time of cells compared to the negative control. This combination also demonstrated a strong synergistic effect, with a CI value of 0.001. The extract as a co-chemotherapy with CIS was capable of increasing the expression of p53, caspase-3, and Bax. The flow cytometry analysis results indicated that HSE could induce apoptosis. Based on the results on the IC_{50} and CI value of HSE, as well as on the doubling time and apoptosis induction of HSE-influenced HeLa cells, it is concluded that HSE has the potential as a co-chemotherapy against cervical cancer.

Keywords: Apoptosis; Co-chemotherapy; HeLa; *Hibiscus sabdariffa* L.; Proliferation

INTRODUCTION

Cancer, being the leading cause of death in every country, significantly decreases life expectancy. It has been reported to be the most prominent cause of death before the age of 70 in 112 of 183 countries in the world, and it is ranked third or fourth in the rest of the countries. The incidence of cancer in the world is expected to increase, with a greater rate in developing countries than in developed countries due to demographic shifts, globalization-related risk factors, and economic expansion (Sung et al., 2020).

Cancer develops as a result of cell proliferation that exceeds apoptosis. Cells can occur in three stages: (1) the initiation by apoptosis-inducing chemicals, (2) the activation of caspase (aspartate-specific cysteine protease), and (3) the release of cellular components (proteolysis) (Bedoui et al., 2020). The regulation of apoptosis involves the Bcl-2, p53, Apaf, and caspase protein families, with anti-apoptotic proteins (Bcl-2 and Bcl-xL), pro-apoptotic proteins (Bax, Bak, and Bad), and BH3 proteins (Bid, Bim, Bik, and PUMA/p53 upregulated modulator of apoptosis) belonging to the Bcl-2 protein family. In cells undergoing apoptosis, there may occur increased expression of pro-apoptotic proteins (Bax, Bid, and Bak), reduced expression of anti-apoptotic proteins (Bcl-2 and Bcl-xL), increased cytochrome c levels, and activation of caspase. In other words, the

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expression of various proteins found in cancer cells influences the many processes of apoptosis. Tracing the expression of proteins involved in the apoptotic mechanism is thus required in the creation of anticancer substances, such as those used in cervical cancer treatments (Haghighi et al., 2023). The mechanism of apoptosis can also be observed with a flow cytometer. Results of testing with a flow cytometer in a previous study showed that cells in the sub-G1 phase were increased by a sample intervention, indicating accelerated apoptosis compared to the negative control (McKinnon, 2018).

In addition to radiation and surgery, cervical cancer can be treated with anticancer drugs, such as cisplatin (CIS), doxorubicin, carboplatin, and hycamtin. However, these medicines have significant toxicity or side effects and lead to poor prognoses since they not only kill cancer cells but also affect normal cells (Yajid et al., 2018). Therefore, alternative anticancer drugs that are selective against cancer cells while not damaging normal cells are urgently needed.

Natural substances with antioxidant and anticancer characteristics can be used as adjunctive medications in cancer prevention and treatment (Ozkan et al., 2019). Specific and non-specific factors must be considered to standardize the extract. Some plants from the genus *Hibiscus* are natural sources of substances with anticancer potential. *Hibiscus calyphyllus*, *Hibiscus deflersii*, and *Hibiscus micranthus*, for example, exhibit anticancer potential in petroleum ether, toluene, dichloromethane, ethyl acetate, and ethanol fractions (Fitmaurice et al., 2018). *Hibiscus sabdariffa* L. (HS) is another *Hibiscus* species that has been reported to demonstrate anticancer activity.

An aqueous extract of HS can reduce the incidence of breast cancer in DMBA (7,12-dimethylbenz(a)anthracene)-induced rats. HSE doses of 125 and 250 mg/kg reduced tumor incidence by 63% and 75%, suppressed tumor burden by 84.86% and 38.78%, and reduced tumor volume by more than 72%, respectively, when compared to the DMBA-induced group. HSE also protected mice from DMBA-induced breast neoplasia, with 125 mg/kg being the most effective dose. It also increased SOD activity while lowering MDA levels. HS exhibited anticancer and antioxidant properties in mice, which are important for cancer treatment (Bassong et al., 2022).

Mishra et al. (2016) described the biosynthesis of ZnO nanoparticles with HSE, which is lethal to cancer cells. In addition, MTT assay was used to test breast cancer (MCF-7), cervical cancer (HeLa), epithelioma (Hep-2), and normal human dermal fibroblasts (NHDF). HS water extract was reported to demonstrate substantial antioxidant and antiproliferative effects on breast cancer cell lines (MCF-7 and MDA-MB-231), ovarian cancer cell lines (Caov-3), and cervical cancer cell lines (HeLa) (Boncler et al., 2014). *In vitro* and *in vivo*, HSE has anticancer and antioxidant properties. HS's anticancer properties are attributable to its bioactive compounds, including flavonols, quercetin, and polyphenols, as well as a range of other significant components related to antioxidant and anticancer activities (Laskar et al., 2020). The flavonoid content of HS flowers can affect numerous signalling pathways, including Bcl-2, via the p53 pathway, which targets apoptosis, inflammation, and oxidative stress, potentially leading to anticancer effects (Rahman et al., 2021).

In various studies, CIS anticancer treatment was paired with brequinor antibiotics (Bamias et al., 2018), trifluoperazine (Kuo et al., 2019), EMA/CO (etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine) (Lybol et al., 2013), gemcitabine (Chen et al., 2020), curcumin (Demir et al., 2016), and tomatoes (Owoeye et al., 2015). The results of these studies showed that combining CIS with other substances could improve the effect of CIS. However, no investigation into HS as a co-chemotherapy with CIS aimed to obtain a synergistic effect by reducing the dose of CIS as an anticancer against HeLa cells has been conducted.

Considering the potential of HSE against cervical cancer cells, it is necessary to investigate its combination with CIS to reduce toxicity in normal cells. Anticancer testing of HSE has recently been carried out on a single sample. This study aimed to further determine the potential of HSE as a co-chemotherapy in increasing the effectiveness of CIS through increased cytotoxic effects and induction of apoptosis in HeLa cervical cancer cells. The results of this study are expected to enhance the benefits of using HSE as a CIS co-chemotherapy agent in the treatment of cervical cancer.

MATERIALS AND METHODS

Materials and cell culture

Hibiscus sabdariffa L. simplicia was received from CV Herbal Anugrah Alam, with the statement letter number 01/009/HAA/IV/2023. HeLa cells were received from the *In Vitro* Cell Culture Laboratory of the Pharmacology Laboratory of Gadjah Mada University's Faculty of Medicine, Public Health, and Nursing. HeLa cells were grown at 5% CO_2 and 37°C in RPMI (Roswell

Park Memorial Institute) media supplemented with FBS (fetal bovine serum), MTT reagent (Rosswell Park Memorial Institute), PBS (phoshate buffer saline), SDS (sodium dodecyl sulfate), Folin-Ciocalteu reagent, and 96% ethanol.

***Hibiscus sabdariffa* L. extraction**

Dry simplicia of HS (7.5 kg) was extracted by maceration with 17.8 L of 96% ethanol solvent in a closed container. The maceration process was carried out for 24 hours, with stirring for one hour. Next, the mixture was filtered, the filtrate was collected, and the solids were re-macerated using the same solvent up to two times. The collected filtrate was then concentrated with a rotary evaporator, followed by evaporation using a water bath at 50°C until a thick extract was obtained (Różyło, 2020).

Specific and non-specific standardization of HSE

Standardization of HSE was carried out with specific and non-specific standardization measurements. Specific standardization included organoleptic and chemical content tests (total phenolic, total flavonoid, and total tannin). Non-specific standardization included tests of specific gravity, gravimetric moisture content, total ash content, acid insoluble ash content, microbial contamination, heavy metal contamination, and solvent residue.

Phytochemical screening tests, including phenolic, flavonoid, tannin, alkaloid, saponin, and terpenoid tests, were also carried out (Klein-Junior et al., 2021). The determination of total phenolic content used the UV-vis spectrophotometry method with the Folin-Ciocalteu reagent at a wavelength of 725 nm. The determination of total flavonoid content used an $AlCl_3$ detector at a wavelength of 438 nm (Formagio et al., 2014). The determination of total tannin content used the UV-vis spectrophotometry method with the Folin-Ciocalteu reagent at a wavelength of 765 nm (Ansori et al., 2021).

Specific gravity testing used a pycnometer, moisture content testing was conducted gravimetrically, microbial contamination testing used the pour plate method, heavy metal contamination testing used atomic absorption spectroscopy (AAS), and solvent residue testing used the Stahl distillation tool according to the Ministry of Health of the Republic of Indonesia (Kemenkes RI, 2000). The testing of total ash content, acid insoluble ash content, gravimetric moisture content also complied with the Ministry of Health of the Republic of Indonesia (Kemenkes RI, 2017).

***In vitro* cytotoxicity and antiproliferation tests against HeLa cells**

In vitro studies of HeLa cells were performed with the ethical approval of the UAD Research Ethics Committee on August 4, 2023, number 0123071177. Cells were inserted in a 96-well plate (each well contained 1.5 x 10^3 cells) and were incubated overnight at 37°C, 5% CO_2. After incubation, extracts at various concentrations (62.5–2,000 µg/mL) were added to each well and were incubated for 24 hours. Subsequently, each well received 10 L of 0.5% MTT in PBS. The reaction was stopped by adding 10% SDS for four hours before intensity measurements were taken. An ELISA reader was used at a wavelength of 595 nm to measure the intensity of the developing purple hue. Information on absorbance was collected (Equation 1) (Artanti et al., 2021).

$$\%\ living\ cells = \frac{(sample\ absorbance - medium\ absorbance)}{(control\ absorbance - medium\ absorbance)}\ x\ 100\%$$

The results of the sample concentration versus cell viability testing were analyzed using a linear regression equation to obtain the IC_{50} value, following the equation y = bx + a. Based on the IC_{50} value obtained from a single cytotoxicity test, concentration series of 1/2 IC_{50}, 1/4 IC_{50}, and 1/8 IC_{50} of both HSE and CIS were prepared. The antiproliferation test was carried out in the same manner as the cytotoxicity test, with incubation periods of 0, 24, 48, and 72 hours. The doubling time as an antiproliferation test parameter was obtained from the correlation graph of incubation time (hours) and cell viability, determined using the formula below (Equation 2) (Eskandari, 2019):

$$Doubling\ time = \frac{Y - A}{B}\ x\ 100\%$$

where Y is log (2 x initial living cell count), A is the intercept, and B is the slope.

Combination index (CI) test

CIS and HSE were used as samples. Tests were carried out based on the IC_{50} results of the samples, which were then combined at the IC_{50} concentration of each sample. The absorbance was used to count living cells affected by each sample according to the following equation (Equation 3):

$$Combination\ Index = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2\ Dx1}\ x\ 100\%$$

where (Dx)1 and (Dx)2 are the concentrations of each substance required to produce an impact (IC_{50} on HeLa cell proliferation), and D1 and D2 are the concentrations of both compounds required to produce the same effect (Artanti et al., 2021).

Immunocytochemistry test

The 1,000 L of sample in the RPMI 1640 growth medium was added to the cell suspension in each individual well (cell density of 5 x 10^5 cells/mL) until the final series level in the well was reached, which was 24.45 g/mL. The 1,000 L of sample was then replaced with 1,000 L of culture medium in the control group. The plate was incubated for 24 hours at 37°C in a 5% CO_2 incubator. The incubated cells were extracted, and smears were produced on poly-L-lysine slides. A light microscope was used to perform immunocytochemistry tests utilizing monoclonal primary antibodies (IgG) targeting p53, Bax, and caspase-3. Cells that express p53, Bax, and caspase-3 proteins produce a brown/dark color; otherwise, a purple/blue color is produced (Roll & Madri, 2018).

Data Analysis

Cytotoxicity tests, selectivity tests, and antiproliferation tests were based on the percentage of living cells determined using Equation 1. The selectivity index against Vero cells was calculated using Equation 4:

$$\text{Selectivity index} = \frac{\text{IC50 on Vero Cells}}{\text{IC50 on HeLa Cells}}$$

HSE and CIS combination tests were performed using the Compusyn application, yielding Combination Index values for all treatments. An analysis of antibody expression in the immunocytochemistry test was performed by comparing the expression of antibodies in sampled and unsampled cells. The percentage of expression was calculated using Equation 5:

$$\text{Percentage of expression} = \frac{\text{Number of cells expressing antibodies}}{\text{Number of living cells}}\ x\ 100\%$$

To determine the effect of HSE on the doubling time and antibody expression in each group, a statistical analysis was carried out with the SPSS 20.0 program. The samples were first subjected to a normality test, a Saphiro-Wilk test, a homogeneity test, and a Levene test. ANOVA was conducted at a significance level of 0.05, followed by the post-hoc least significant difference (LSD) test.

RESULTS

***Hibiscus sabdariffa* L. extraction**

As shown in Figure 1, the HSE yield was around 15.87%. The organoleptic observation results showed that HSE was a viscous red extract with a distinctive odour and a sour taste. These properties met the requirements laid down in the second edition of the Indonesian Herbal Pharmacopoeia (Kemenkes RI, 2017).

(A) (B) (C)

Figure 1. (A) *Hibiscus sabdarifa* L., (B) powder, and (C) extract

Phytochemical screening of HSE

HSE was subjected to phytochemical screening. Phytochemicals are composed of phenolics, flavonoids, tannins, alkaloids, saponins, and terpenoids. HSE was found to be positive for containing phenolics, flavonoids, tannins, saponins, and terpenoids. The phytochemical contents identified in HSE are presented in Table I.

Specific and non-specific parameter tests

The specific parameters examined included total phenolics, total flavonoids, and total tannins of HSE, as shown in Table II. Meanwhile, the non-specific parameters examined comprised the following: 1) specific gravity, 2) ash content, 3) acid-insoluble ash content, 4) water content, 5) microbiological and heavy metal contamination, and 6) residual solvent. The non-specific standardization test results met the requirements laid down in the second edition of the Indonesian Herbal Pharmacopoeia.

***In vitro* cytotoxicity test against HeLa cells**

As shown in Table III, HSE and CIS exhibited cytotoxic effects on HeLa cells, with IC_{50} values of 32.35 ± 3 µg/mL and 2.91 ± 0.4 µg/mL, respectively. The cytotoxicity test against Vero cells yielded IC_{50} values of 6,779.06 ± 229.44 µg/mL and 8,106.7 ± 675.73 µg/mL for HSE and CIS, respectively.

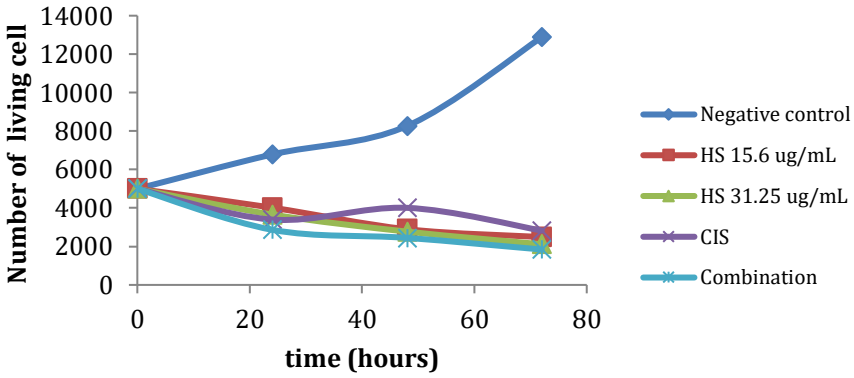


Figure 2. The number of living cells after incubation for 0, 24, 48, and 72 hours, following induction by HSE, CIS, and HSE-CIS combination

Table I. Phytochemical screening of HSE

Phytochemical compounds	Test	Observations (Positive Test Indication)	Result
Phenolics	Ferric chloride	A dark green color	+
Flavonoids	NaOH	A green-yellow color	+
Tannins	Gelatine	A white precipitate	+
Alkaloids	Dragendroff	Non-formation of a reddish-brown precipitate	-
Saponins	Foam	Formation of a 1-cm-thick layer of foam	+
Terpenoids	Liebermann- Burchard	A red-brown color	+

Description: (+): Positive test; (-): Negative test

Combination index test

A combination index (CI) test was conducted to assess the interaction mode between HSE and CIS. The interpretation of the CI values of the HSE and CIS combination against HeLa cells is presented in Table IV. This table revealed a very strong synergistic effect of the HSE and CIS combination, with the best CI value being 0.001, achieved from the use of HSE and CIS at concentrations of 1,168 µg/mL and 3,035 µg/mL, respectively. A CI value < 0.1 indicates a very strong synergistic effect.

Apoptosis test with immunocytochemistry

The antiproliferation test results are presented as doubling time, which is the time required for cells to double their original number. The doubling time of HeLa cells influenced by HSE, CIS, and their combination was much longer compared to that of control cells (Figure 2), indicating that HSE, CIS, and their combination had antiproliferative activity. The doubling time results are shown in Figure 2.

The molecular mechanisms associated with apoptosis induction by HSE in HeLa cervical cancer cells and the expression levels of p53, Bax, and caspase-3 were examined by immunocytochemistry. HeLa cells treated with supernatant from isolated HSE at 45 µg/mL showed a significant increase in mRNA expression compared to the control (untreated HeLa cells), with $p < 0.05$. The results are shown in Figure 3.

Overexpression of pro-apoptotic genes, according to earlier research, prevents the

Table II. Specific and non-specific parameters of HSE

Parameters	ẍ ± SD
Specific:	
Phenolics (µg/mL)	105.346 ± 0.236
Flavonoids (µg/mL)	16.477 ± 0.124
Tannins (µg/mL)	86.335 ± 0.105
Non-specific:	
Specific gravity (g/mL)	1.050 ± 0.000
Total ash content (%)	0.799 ± 0.02
Acid-insoluble ash content (%)	0.450 ± 0.077
Water content (%)	8.342 ± 5.355
Total plate count (colonies/g)	< 1 x 10^1 ± 0.000
Yeast most count (colonies/g)	< 1 x 10^1 ± 0.000
Cd content (ug/g)	Undetectable
Pb content (ug/g)	0.201 ± 0.065
Solvent residue	None

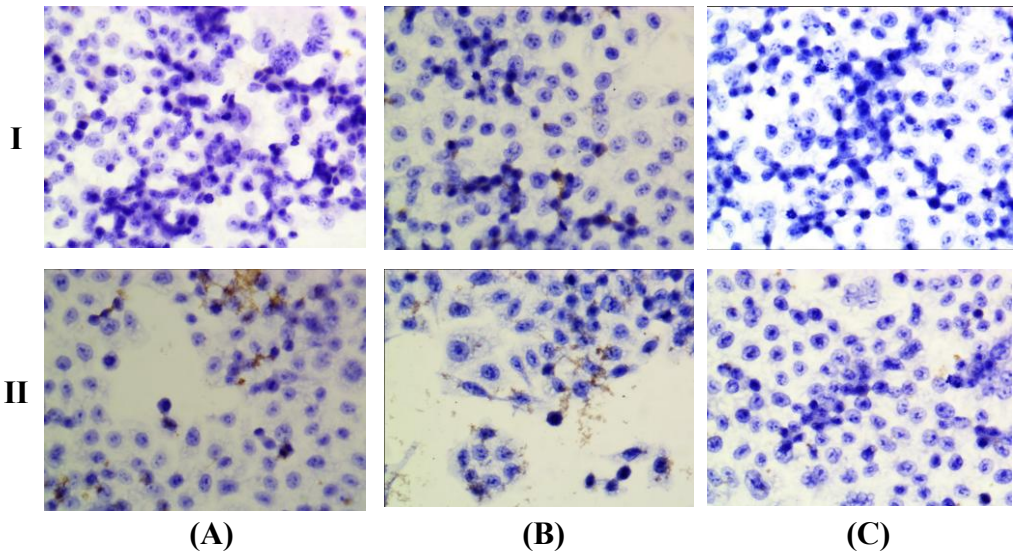


Figure 3. Expression of the p53, Bax, and caspase-3 genes in HeLa cells, following the induction by HSE: (A) p53; (B) Bax; and (C) Caspase-3. I: Negative control; II: Induced by HSE.

Table III. IC_{50}, doubling time, and expression p53, Bax, and caspase-3 following the induction by HSE and CIS

Sample	IC_{50} (µg/mL)	Doubling time (hour)	Expression (%)		
			p53	Bax	Caspase-3
HSE	32.35 ± 3.0				
HSE 31.25 µg/mL	NA	-131.88 ± 5.53	138.92 ± 3.04	19.88 ± 2.15	16.55 ± 3.4
HSE 15.6 µg/mL	NA	-141.02 ± 10.65	NA	NA	NA
CIS	2.91 ± 0.4	-221.07 ± 3.21	66.21 ± 4.79	12.89 ± 2,41	14,96 ± 0.56
Combination of HSE-CIS	NA	-134.72 ± 2.17	75.92 ± 2.85	32.93 ± 5.82	28.06 ± 2.05
Negative control	NA	54.48 ± 9.42	58.7 ± 1.79	10.73 ± 1.87	10.44 ± 1.2

*NA: Not available

Table IV. Combination Index of HSE and CIS against HeLa cells

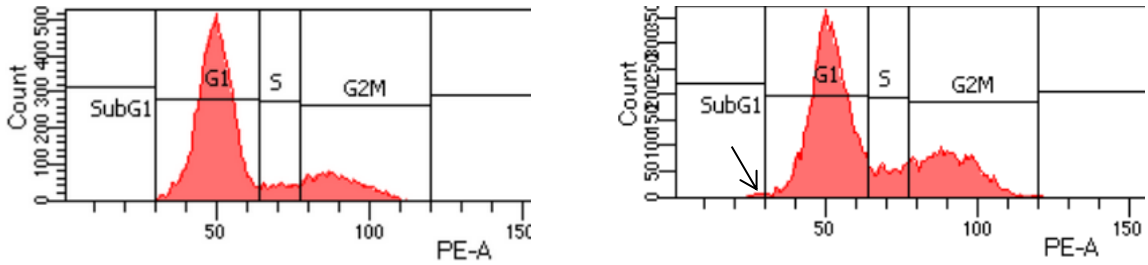
Sample		CIS (µg/mL)			
		9	3	1.5	0.7
HSE (µg/mL)	74	10.550	0.195	1.29	8.9
	32	21.564	0.012	2.61	1.7
	16	10.233	0.203	1.44	1.04
	8	6.327	0.435	**0.001**	1.81

Description: HSE: *Hibiscus sabdarifa* extract; CIS: Cisplatin

development of cancer cells. Additionally, activated caspase-3 (execution) and caspase-8 (initiator) are crucial biomarkers for detecting cell apoptosis. In HeLa cells exposed to HSE, CIS, or a combination of the two, pro-apoptotic proteins such as p53, Bax, and caspase-3 were found to be expressed (Table III).

Compared to either therapy applied independently, the combination of HSE and CIS increased the expression of the p53 and caspase-3 proteins in HeLa cells. In contrast to single CIS therapy, the Bax protein expression was higher when HSE and CIS were combined. This finding is corroborated by the ANOVA results, which revealed significant differences between the three groups and between the administration of CIS, HSE, and their mixtures.

The flow cytometry analysis results presented in Figure 4 showed that there was an increase in cells in the sub-G1 phase. This shows that HSE could induce HeLa cell apoptosis, as the cells were headed for the arrest phase.



(A) **(B)**

Figure 4. HeLA cell cycle based on flow cytometry, following the induction by (A) negative control and (B) HSE

Polyphenolic substances, including flavonoids, have been shown to induce apoptosis in various cancer cell lines (Rauf et al., 2022). Tannins also promote cell cycle arrest by raising p27 protein expression, which produces cell cycle hurdles in the G2/M phase. Inhibiting DNA topoisomerase I/II activity, increasing p53, decreasing ROS (reactive oxygen species), regulating heat shock protein expression, modulating the apoptotic pathway, activating caspase-9 and caspase-3, and decreasing the expression of nuclear transcription factors are all mechanisms for inducing apoptosis. Sarkar et al. (2009) reported the activation of NF-κB and endonuclease, as well as the reduction in Mcl-1 protein.

DISCUSSION

Phytochemical screening of HSE

Based on Table I, HSE was positive for containing phenolics, flavonoids, tannins, saponins, and terpenoids. This result is consistent with previous research by Bariyyah et al. (2019) and Suniarti et al. (2022), which also found phenolics, flavonoids, tannins, saponins, and terpenoids in HSE.

Specific and non-specific parameter tests

The specific standardization test results presented in Table II showed that phenolics were a dominant constituent in HSE compared to flavonoids and tannins. This finding is in line with previous research by Djaeni et al. (2017) and Chatepa et al. (2023). For the non-specific standardization test, a pycnometer was used to assess the density of HSE. This test aimed to offer an overview of the chemical composition dissolved in HSE. It was found that the density of HSE was 1,050 ± 0.000 µg/mL.

An ash content test was also conducted to offer an overview of the internal and external mineral contents of HSE from the beginning of the process until the extraction phase (Kemenkes RI, 2000). It was found that HSE had a total ash level of 0.799 ± 0.02%. This ash content met the requirements laid down in the second edition of the Indonesian Herbal Pharmacopoeia as it remained below the recommended 5.6% threshold. To ascertain the contamination of acid-insoluble minerals or metals in a product, acid-insoluble ash content was measured. In this study, HSE was found to contain 0.450 ± 0.077% acid-insoluble ash. This level of acid-insoluble ash met the requirements laid down in the second edition of the Indonesian Herbal Pharmacopoeia too as it did not exceed the recommended 1.4% limit (Kemenkes RI, 2017).

The determination of water content based on the gravimetric method was carried out by oven-heating the sample to remove the water content (Balekundri & Mannur, 2020). The water content of HSE in this study was found to be 8.342 ± 5.355%. As the second edition of the Indonesian Herbal Pharmacopoeia recommends that water content should not exceed 10%, the water content of HSE in this study was considered to have met the requirements (Kemenkes RI, 2017). An assessment of water content was conducted to avoid microbial contamination and prevent chemical instability (Govindaraghavan & Sucher, 2015).

Atomic absorption spectrophotometry (AAS) was used to evaluate the heavy metal contamination level. This method did not detect Cd content in HSE, but it detected Pb at a level of 0.201±0.065 µg/g. In other words, the requirements that the Pb content and Cd content should be below 10 µg/g and below or equal to 0.3 µg/g, respectively, were met (BPOM, 2014).

The extract purity was also examined by calculating the total plate number (colonies/g) for bacterial contamination and the yeast fungi number (colonies/g) for mould contamination (Govindaraghavan & Sucher, 2015). The test aimed to ensure that the HSE under study was pure from any microbial and fungal contamination (Ministry of Health of the Republic of Indonesia,

2000). Table II shows that the bacterial and mould levels in the HSE met the requirements, as was found in previous research by Elhassan et al. (2014) and Effendy et al. (2024). According to the rule of the Head of the National Agency of Drug and Food Control of the Republic of Indonesia on the Quality Requirements for Traditional Medicines, the upper limit for bacterial contamination is 10,000 colonies/g, while the upper limit for mould contamination is 1,000 colonies/g (BPOM, 2014). To ensure that there was no residual solvent in the product during the extraction process, a solvent residue test was carried out (Kemenkes RI, 2000), and the results showed that the extract was free from any residual ethanol solvent.

***In vitro* cytotoxicity test against HeLa cells**

A cytotoxicity test against cancer cells is a basic test commonly used to assess anticancer drugs and chemopreventive compounds. IC_{50} is the parameter used to determine the potential of the tested compounds, and one of the cytotoxicity testing methods widely used *in vitro* is the MTT method (Sukardiman, 2014). This method is commonly used to evaluate cell viability and screen for antiproliferative agents against cancer cells (Boncler et al., 2014; Eskandari, 2019).

Based on the National Cancer Institute (NCI), cytotoxicity levels can be classified into four categories: high cytotoxicity if IC_{50} < 20 µg/mL, moderate cytotoxicity if the IC_{50} ranges from 21 to 200 µg/mL, weak cytotoxicity if the IC_{50} ranges from 201 to 500 µg/mL, and zero toxicity if IC_{50} > 500 µg/mL (Damasuri et al., 2020). In this study, the IC_{50} value of the HSE was categorized as moderate. This suggests that HSE could be utilized to prevent and inhibit the growth of cancer cells. This finding is in accordance with previous research, which reported HSE's cytotoxic activity against T47D breast cancer cells, with an IC_{50} value of 32.3 ± 2.15 µg/mL (Edityaningrum et al., 2024).

The selectivity index values for HSE and CIS against Vero cells and HeLa cells were 209 and 2,785, respectively, indicating that both HSE and CIS had high selectivity against HeLa cells. A parameter of more than 3 indicates high safety on normal cells (Elizondo-Luévano, 2022). Depending on the dose, HSE could suppress cell growth (Figure 2), as evidenced by a decrease in HeLa cell viability following HSE treatment. When tested at higher doses, HSE outperformed the other two samples.

The cytotoxic activity of HSE against HeLa cells stems from the chemical compounds contained in HSE, including anthocyanins, flavonoids, quercetins, polyphenols, and saponins. Anthocyanins can interact with proteins from the Bcl family and inhibitors of apoptosis protein family to activate the apoptosis response through the caspase-dependent cascade (Charepalli et al., 2016). They can induce mitochondria to release endonuclease G and apoptosis-inducing factors through the JNK pathway to trigger caspase-independent apoptosis of the LNCaP and PC-3 prostate cancer cell lines (Liu et al., 2016). Their antitumor activity is related to antioxidant, anti-inflammatory, and anti-proliferative properties, as well as their ability to regulate apoptosis-related mediators, including p53, Bcl-2, Bax, cytochrome c, and caspase-3, in several cancer cell models (Chen et al., 2022). In the prostate cell lines DU145 and LnCap, cyanidin determines caspase-3 activation and DNA fragmentation (Sorrenti et al. 2015). In U87 glioblastoma cells, apoptosis occurs through Bax, accompanied by increased p53 mRNA expression and decreased Bcl-2 levels (Hosseini et al., 2017). Anthocyanins, in the form of cyanidin, induce apoptosis through the upregulation of PPARγ and Bax mRNA expression in osteosarcoma cell lines (Atashi et al., 2020).

Flavonoids can induce apoptosis in HL-60 leukemia, stimulated by the release of cytochrome c to the cytosol, procaspase-9 processing, and caspase-3-dependent mechanisms. Meanwhile, the induction of apoptosis by flavonoids is attributed to their cancer chemopreventive activity (Fallah et al., 2017). Quercetin can specifically induce apoptosis of human HeLa cells, in which case the mechanism of apoptosis induction is associated with the activation of Caspase-3 and Caspase-8. Caspase-3 is an execution protein effector molecule in the apoptotic cascade signalling pathway (Su et al., 2016).

The anticancer activity of polyphenols, meanwhile, is attributed to their antioxidant activity as potent radical scavengers, metal chelators, modifiers of endogenous defense mechanisms, which involve components such as SOD, CAT, GPx, and GSH, and regulators of several proteins and transcription factors, such as Nrf2 (Rudrapal et al., 2022; Suraweera et al., 2020). In addition, polyphenols can also reduce tumor cell growth by inhibiting the biosynthesis of polyamines and signal transduction enzymes, such as PTK, PKC, and PI3K, and by modulating the expression of proteins involved in cell cycle arrest, cell migration, metastasis, and cell death (Chairez-Ramirez et al., 2021; Janabi et al., 2020;

Sharma et al., 2022; Abotaleb et al., 2019; Hazafa et al., 2020; Hazafa et al., 2022)

Combination index test

Table IV indicates that the cytotoxic effect of the combination of HSE and CIS was more significant than the sum of the effect of HSE and CIS in isolation. This result is credited to the phytochemical compounds contained in HSE, such as flavonoids and saponins. The combination of CIS and flavonoids induces the depletion of cellular oxidative machinery, helps mitochondria to dysfunction, and triggers apoptosis. Flavonoids have been reported to have considerably boosted the efficacy of CIS treatment in comparison to the use of CIS as a single treatment of gastric cancer (Ghavami et al., 2020). A biological mechanism exists in the CIS and HSE combination, which guards against nephrotoxicity by addressing deficiencies in p53, multi-gene-activated protein kinase (MAPK), and protein kinase B (AKT), which are important for preventing apoptosis. Research also highlights that saponins can protect against nephrotoxicity that is induced by CIS. Saponins contributes to cell resistance by sensitizing CIS through the induction of apoptosis in ovarian cancer (Ju et al., 2015; Wang et al., 2018).

The synergistic increase in the cytotoxic effect due to the application of the HSE and CIS combination was investigated through antiproliferation and apoptosis studies. The cytotoxic effect can be increased by promoting apoptosis in HeLa cells. Therefore, to prove indications that the combination of HSE and CIS can promote apoptosis, an immunocytochemistry analysis was performed for the proteins involved in the mechanism of apoptosis.

Apoptosis test with immunocytochemistry

Phenolics, flavonoids, and tannins in HSE at concentrations as shown in Figure 2 induced apoptosis in HeLa cells. Flavonoids, in particular, have the ability to boost p53 expression, in which case the p53 gene causes mitochondria to release cytochrome c into the cytoplasm and activates caspases, resulting in cell death or apoptosis (Nurani et al., 2020).

Observations of the expression of apoptosis regulatory proteins using the immunocytochemical method and following the principle of specific antibody binding were carried out. Binding of apoptotic proteins is indicated by cytoplasmic staining to brown. The expression of p53, Bax, and caspase-3 as pro-apoptotic proteins will increase when apoptosis is induced, resulting in a more intense brown color. Treatment using HSE, CIS, or a combination of both against HeLa cells showed a more intense brown color in the cytoplasm compared to control.

Cervical cancer cells are caused by infection with HPV (Human papillomavirus), which can produce the E6 oncoprotein. E6 binds the tumor suppressor protein p53, thereby enabling HeLa cancer cells to avoid apoptosis. However, the results of this study showed that HSE could increase the expression of p53 in HeLa cells and thus increase the occurrence of the apoptotic process in HeLa cells. This ability to induce apoptosis may be related to the phenolic, flavonoid, and tannin contents in HSE (Yuliastri et al., 2022).

CONCLUSION

HSE contains phenolics, flavonoids, tannins, saponins, and terpenoids. HSE and CIS co-chemotherapies could inhibit the growth of HeLa cervical cancer cells, demonstrating cytotoxic, antiproliferative, and apoptosis inductive effects. HSE showed the ability as a co-chemotherapy with a very strong synergistic effect, particularly at the 8 µg/mL HSE and 1.5 µg/mL CIS dose. The mechanism by which the combination of HSE and CIS inhibits the growth of HeLa cells involves apoptosis, which is mediated by the induction of p53, caspase-3, and Bax expression.

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