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Research Article

Anticancer Activity Studies of Self-nanoemulsifying Drug Delivery Systems (SNEDDS) *Hibiscus sabdariffa* L. as Cisplatin Co-Chemotherapy in T47D Breast Cancer Cells

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ABSTRACT

Hibiscus sabdariffa L. (HS) exhibits strong anticancer activity, which can potentially treat breast cancer. A delivery system, namely self-nano-emulsifying drug delivery systems (SNEDDS), has been developed by our research group to carry the HS, indicating cytotoxicity against T47D breast cancer cells. To further evaluate the anticancer activity of HS SNEDDS, this study aimed to measure the anticancer activity of HS SNEDDS as a Cisplatin co-chemotherapy agent. The potential of HS SNEDDS as an anticancer agent was evaluated by measuring the anti-proliferation, combination index (CI), and immunocytochemistry of Tumor Necrosis Factor-α (TNF-α) gene expression. HS SNEDDS at a concentration of 241.990 µg/mL exhibited anti-proliferation properties against T47D cells with the highest doubling time, i.e., 9.4-fold compared to the control. The combination of HS SNEDDS and Cisplatin indicated a lowest CI of 0.17, showing the synergistic effect. Furthermore, combining HS SNEDDS and Cisplatin showed the highest TNF-α gene expression of 17.939 ± 7.323, indicating a better potency as a Cisplatin co-chemotherapy agent in T47D breast cancer cells compared to control and single administration. Based on these results, HS SNEDDS can be used as a co-chemotherapy agent for Cisplatin, which can increase the Cisplatin effect.

INTRODUCTION

Breast cancer represents a significant global health issue, being the most frequently diagnosed cancer among women, with approximately 2.3 million new cases and over 685,000 fatalities recorded in 2020 (Nolan *et al.*, 2023). Accordingly, breast cancer treatment is an important step toward minimizing the global growth in breast cancer. Breast cancer can be treated with chemotherapy, surgery, and radiation oncology. Chemotherapy is the primary treatment option because it effectively targets cancer cells, either to cure post-operative disease, shrink tumors before surgery, or delay progression in metastatic cases, and it can be combined with immunotherapy or targeted therapy (Trayes and Cokenakes, 2021). One of the treatments for breast cancer is chemotherapy with Cisplatin, which is one of the first-line chemotherapy agents for breast cancer (Wang *et al.*, 2022). However, Cisplatin often faces obstacles, such as significant side effects, drug

resistance, and toxicity to normal cells (Aldossary, 2019). In order to avert these challenges, a combination therapy approach involving co-chemotherapy agents from natural ingredients with potential synergism is an alternative to increase the effectiveness of treatment. The development of natural ingredients, namely *Hibiscus sabdariffa* L (HS), incorporated into the self-nanoemulsifying drug delivery systems (SNEDDS) as a carrier of the drug has been previously carried out by our research group. Therefore, this study aimed to investigate the anticancer activity of HS SNEDDS as a Cisplatin co-chemotherapy agent.

Hibiscus sabdariffa L HS contains active ingredients indicating pharmacological activities, such as anticancer agency (Anggi *et al*., 2023; Hassan *et al*., 2016; Tsai *et al.*, 2016). The active ingredients are flavonoids, polyphenols, and anthocyanins, which are known to induce apoptosis, inhibit cancer cell proliferation, and modulate molecular signaling pathways, including the expression of genes involved in inflammation and apoptosis, such as tumor necrosis factor-alpha (TNF-α) (Laskar and Mazumder, 2020). The expression of TNF-α in the cancer microenvironment has a dual role, namely as a pro-inflammatory mediator that can worsen cancer development or as a proapoptotic agent that supports cancer cell apoptosis (Diaz Arguello and Haisma, 2021). According to research conducted by Hamza *et al*. (2023), administering a dose of HS extract of 500 mg/kg/day either before or after administration of 10 mg/kg cisplatin can reduce hepatotoxicity in male Wistar albino rats by decreasing the enzyme activity, including alanine and aspartate aminotransferase causing the liver damage as well as necrosis in liver tissue of rats receiving Cisplatin treatment. In the *in vitro* studies, HS extract has also been shown to be toxic to breast cancer cells (Wu *et al*., 2016), lung (Fithrotunnisa *et al*., 2020), and human squamous cell carcinoma (HSCC)(Akram *et al*., 2024) by increasing the levels of reactive oxygen species in cancer cells, resulting in cell death. However, light, pH, and temperature affect the stability of phenolic and anthocyanin compounds in the HS extract during storage. Studies conducted by Paraíso *et al*. (2020) exhibited that the concentration of anthocyanins and total phenolics in HS extract decreased by 25% and 23%, respectively after a storage period of 22 days at 25 °C, whereas, after the storage for 22 days with the exposure to light at 25 °C, the levels of anthocyanins and total phenolics decreased significantly by 41.95% and 38.02%, respectively. Therefore, incorporating HS into SNEDDS becomes a challenging strategy to protect the active substance from unfavorable environments, leading to the maintenance of the anticancer activity (Azizi *et al*., 2022).

In this research, anticancer activity studies were conducted to continue a previous study, namely the development of SNEDDS as a carrier of HS. The experiments in this study included the anti-proliferation, combination index (CI), and immunocytochemistry studies on TNF-α gene expression. The results regarding anticancer activity studies indicated that HS SNEDDS could potentially be used as a co-chemotherapy agent for Cisplatin, which can increase the effect of Cisplatin and be safe for normal cells.

MATERIALS AND METHODS

Materials

T47D cells were obtained from the In Vitro Cell Culture Laboratory of the Parasitology Laboratory of the Medicine Faculty, Public Health and Nursing, Universitas Gadjah Mada. T47D cells were stored in Rosewell Park Memorial Institute (RPMI) media and supplemented with fetal bovine serum (FBS). Cisplatin was purchased from PT Kalbe Farma. Paramount Detection Kit originated from Bio Gear. MTT Reagent was obtained from Roswell Park Memorial Institute. Methanol was purchased from PT Brataco. Additionally, Sodium Dodecyl Sulfate (SDS) was obtained from PT Merck.

Methods

Anti-proliferation study

This study was conducted to evaluate the anti-proliferation ability of HS SNEDDS. SNEDDS is a lipid-based drug delivery system composed of oil, surfactants, and cosolvents/cosurfactants used as drug carriers. In the case of this research, SNEDDS contains *Hibiscus sabdarifae* (HS) as an active ingredient. For this study, KEP UAD granted ethical authorization on July 29, 2024, with the number 012407197. Briefly, a 96-well microplate was filled with cells at a density of 2 × 10^4 cells per well. A 5% liquid CO2 concentration at 37^0C was used for a 24-hour incubation period (model: CO2 Incubator: Hera Cell, United States). Each well was filled with a series of concentrations consisting of 100%, 50%, and 25% of the IC_{50} value, for both HS SNEDDS and Cisplatin, with variations in incubation time for 0, 24, 48, and 72 hours (Hasibuan and Sumaiyah, 2019). Afterward, the wells were incubated for 24 hours, followed by adding 100 µL of 0.5% MTT in PBS, and the wells were incubated for four

hours. At the end of the incubation, 10% SDS was added, and the samples were kept at room temperature in the dark for 24 hours. The produced purple color was measured using an ELISA Reader, at a wavelength of 595 nm (Edityaningrum *et al*., 2024). To calculate the doubling time, the time required for a cell to replicate itself, the equation shown in Eq. 1 was used. The linear regression resulted from the calculation based on data of incubation time (x-axis) and log cell viability (y-axis).

$$x = \frac{Y - A}{B} \quad \text{(Eq. 1)}$$

Y = log 2x initial number of living cells
A = intercept
B = slope

Combination index study

A 96-well plate containing 100 µL of cells was incubated for 24 hours to allow the cells to attach to the wells and adapt to assess the combination index. Subsequently, each well was filled with 50 µL of HS SNEDDS and Cisplatin at concentrations of IC_{50}, ½ IC_{50}, ¼ IC_{50}, and 1/8 IC_{50}, followed by incubating for 24 hours. The combination ratio used in this study was 1:1. Afterward, 100 µL of MTT reagent was added to each well and incubated for 4 hours until formazan was formed. At the end of the incubation, each well was filled with 10% SDS as a stopper, and the samples were kept at room temperature in the dark for 24 hours. The produced purple color was measured using an ELISA Reader, at a wavelength of 595 nm (Chatran et al., 2018). The combination index value between HS SNEDDS and Cisplatin was calculated using Compusyn Version 1.0 developed by Ting Chao-Chou and Nick Martin.

Immunocytochemistry study

Cells were grown on glass coverslips in a 24-well microplate at $5x10^3$ cells/well density. The wells were filled with samples, including HS SNEDDS, Cisplatin, and a combination of Cisplatin and HS SNEDDS. A control was used in this experiment; all of the samples were incubated for 24 hours, followed by adding PBS to rinse the cells before adding the methanol. Afterward, the cells were incubated for ten minutes after being dropped with prediluted blocking serum. Subsequently, the primary monoclonal antibody TNF-α was added. TNF-α gene expression was indicated by the produced brown or dark cells, measured using an Optilab light microscope with five display screens (Imanuel *et al*., 2021). To determine the percentage of TNF-α expression, Eq. 2 was used by comparing antibody expression in samples and control cells, allowing

$$\frac{\textit{Number of cells expressing antibody}}{\textit{Number of living cell}} \times 100\%$$

measurement of antibody expression.

(Eq. 2)

Data analysis

Statistical data analyses were conducted using SPSS Statistics 23 (IBM Corp., Chicago, USA) with the One-Way ANOVA – Post Hoc Tukey test to compare the treatment and control groups. A difference was deemed significant if the *p* value was less than 0.05.

RESULTS AND DISCUSSION

Anti-proliferation study

An anti-proliferation study was conducted to determine the ability of HS SNEDDS to inhibit cell proliferation by extending the time required for cells to duplicate themselves, namely doubling time (Fiorentino *et al*., 2018). This study used a low FBS concentration (0.5%) to minimize growth stimulation in T47D breast cancer cells. This condition aims to reduce cell proliferative activity, thereby encouraging cells to enter the G0 phase, a resting phase outside the active cell cycle. This synchronization is important to align the growth starting point of the entire cell population; therefore, the antiproliferative effect of the HS SNEDDS formulation can be more accurately observed at the rate of cell re-entry into the proliferation cycle. The concentration of HS SNEDDS used in the proliferation study was IC_{50}, and two concentrations below IC_{50}, including half and a quarter of IC_{50}, to avoid cell death during the incubation for 72 hours.

Figure 1 indicates the cell growth of the treatment and control groups during 72 hours of the experiment. The control group showed an increase in cell growth because there was no treatment with the test compound, which means that the cells could adapt to the environment, and the longer the incubation time, the greater the number of living cells. The treatment group experienced a rapid decrease in cell growth at 24 hours. It is suspected that the cells experienced a lag time, describing the adaptation phase. At 48 hours, all treatment groups experienced an increase in cell growth, whereas at 72 hours, it decreased again. It is likely due to the arrest mechanism (cell cycle stops) leading to the death of cells, which undergo apoptosis and stop reproducing themselves (Dona *et al*., 2016).

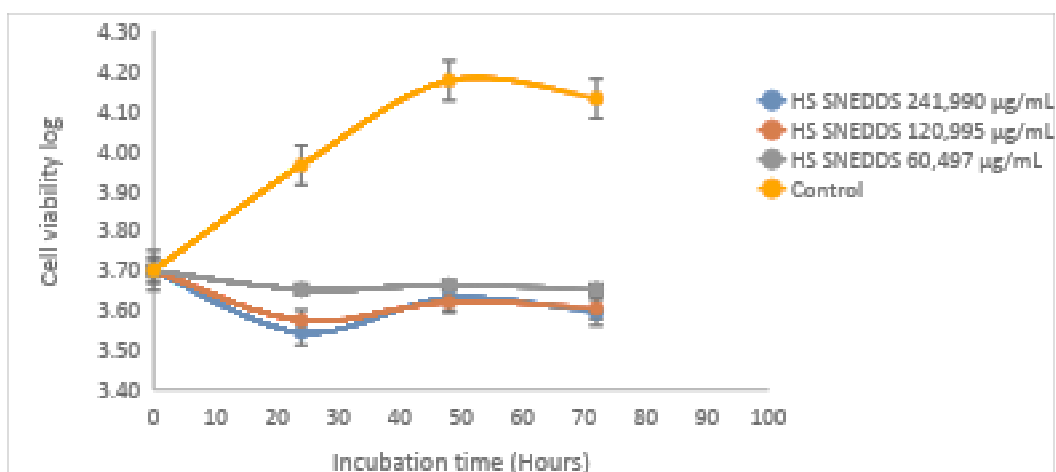


Figure 1. The cell growth of the treatment (HS SNEDDS in various concentrations) and control groups during the 72 hours of the experiment. Data are presented as mean ± standard deviation (n = 3).

Table 1. Doubling Time of Various Concentrations of HS SNEDDS and Control Based on Linear Regression Equation

Concentrations of HS SNEDDS	***Doubling time* (Hours)**
241.990 µg/mL	349.2
120.995 µg/mL	340.2
60.497 µg/mL	314.3
Cell control	37.13

Doubling time is the time needed for the cell to split to perform the proliferation; the lower the doubling time, the higher the number of cells that split. Based on Table 1, the control shows the lowest doubling time compared to others, which is due to there being no HS SNEDDS in the cell culture, which could prevent the T47D cell proliferation. The higher the HS SNEDDS concentration, the higher the doubling time, which is likely caused by the higher ability of HS SNEDDS to decrease cell proliferation (Niu *et al.*, 2024). HS containing active compounds such as anthocyanins, flavonoids, saponins, tannins, and polyphenols can stop the growth of cancer cells by triggering apoptosis and cell cycle arrest (Yasmin et al., 2023). HS inhibits breast cancer cell growth due to its antioxidant properties, reducing tumor incidence, enhancing antioxidant enzyme activities, namely, superoxide dismutase (SOD), while decreasing oxidative stress markers such as malondialdehyde (MDA), thus protecting against cancer progression (Bassong *et al.*, 2022). In another study, HS also indicated the ability to inhibit the proliferation of another breast cancer cell, namely MCF-7 cells. The mechanism is by reducing proteasome activity, increasing autophagy, and inducing translocation of Estrogen Receptor α (ERα) in MCF-7 cells and causing decreased expression of ERα, BRCA1, and caveolin1 in MCF-7(ERα +) and MDA-MB-231 (triple negative) cells. which ultimately interferes with the survival and proliferation of breast cancer cells (Malacrida *et al.*, 2022).

Combination Index (CI) Study

The combination index study was conducted based on data from the IC_{50} of HS SNEDDS and Cisplatin obtained from a previous study. The concentration variations used in this test were 100%, 50%, and 25% of the IC50 value: IC_{50}, ½ IC_{50}, ¼ IC_{50}, and 1/8 IC_{50}. This study was carried out to determine the magnitude of the cytotoxic effect of the combination of HS SNEDDS and Cisplatin on T47D cancer cells and to reduce

Table 2. Cell viability percentage of T47D in the combination of HS SNEDDS and Cisplatin. Data are presented as mean ± standard deviation (n = 3).

Samples	Concentrations (µg/mL)	Cell viability (%)
HS SNEDDS	241.99	52.01± 4.25
	120.995	61.26 ± 1.36
	60.50	84.38 ± 1.92
	30.25	97.09 ± 1.44
Cisplatin	6.269	50.84 ± 1.69
	3.135	56.05 ± 3.27
	1.567	60.13 ± 1.31
	0.784	62.12 ± 4.25
Cisplatin 6.269	SNEDDS 241.99	35.72 ± 4.25
	SNEDDS 120.995	37.91 ± 4.25
	SNEDDS 60.50	37.24 ± 4.25
	SNEDDS 30.25	38.35 ± 4.25
Cisplatin 3.135	SNEDDS 241.99	49.13 ± 4.25
	SNEDDS 120.995	50.73 ± 4.25
	SNEDDS 60.50	52.15 ± 4.25
	SNEDDS 30.25	58.06 ± 4.25
Cisplatin 1.567	SNEDDS 241.99	39.32 ± 4.25
	SNEDDS 120.995	40.27 ± 4.25
	SNEDDS 60.50	41.65 ± 4.25
	SNEDDS 30.25	43.26 ± 4.25
Cisplatin 0.784	SNEDDS 241.99	68.60 ± 4.25
	SNEDDS 120.995	70.91 ± 4.25
	SNEDDS 60.50	73.74 ± 4.25
	SNEDDS 30.25	75.04 ± 4.25

Table 3. Combination Index (CI) of various HS SNEDDS and Cisplatin combination concentrations on T47D Cells.

	Cisplatin (µg/mL)				
		6.269	3.135	1.567	0.784
HS SNEDDS (µg/mL)	241.990	0.84	2.69	1.22	11.35
	120.995	0.55	1.57	0.64	6.7
	60.498	0.26	0.93	0.37	5.5
	30.249	0.17	0.67	0.21	3.19

the risk of using the combination. The cell viability percentage of T47D cells in the combination of HS SNEDDS and Cisplatin, with the MTT method, can be seen in Table 2.

The CI data was obtained using the Compusyn Version 1.0 software application by Ting Chao-Chou and Nick Martin. The results of the CI from Compusyn are presented in Table 3.

The CI value of anticancer drug combinations is useful information for selecting the drug combination to reduce cytotoxicity in normal cells and achieve high efficacy in cancer models, thereby improving treatment outcomes (Kashif *et al.*, 2015). The CI value defining synergism (CI < 1), additive effects (CI = 1), or antagonism (CI > 1) can be simulated immediately and accurately

with CompuSyn software (Chou, 2018). In 16 samples of cytotoxicity studies, the results showed that HS SNEDDS combinations with Cisplatin had a range of index values, from antagonistic to synergistic effects (Table 3). The combination of Cisplatin and HS SNEDDS is synergistic, as evidenced by the smallest CI of 0.17 in the optimal combination of Cisplatin and HS SNEDDS by 30.249 and 6.269 µg/mL, respectively. This combination indicated a significant difference ($p<0.05$) compared to other combination variations. A synergistic drug combination uses two drugs simultaneously, producing a more significant therapeutic effect than the drugs alone (Haryanti *et al.*, 2019). Thus, combining HS SNEDDS and Cisplatin can be a promising anticancer agent, providing a more effective therapeutic effect. Furthermore, the dose of Cisplatin is expected to decrease during the treatment, thereby reducing the risk of resistance and side effects of Cisplatin.

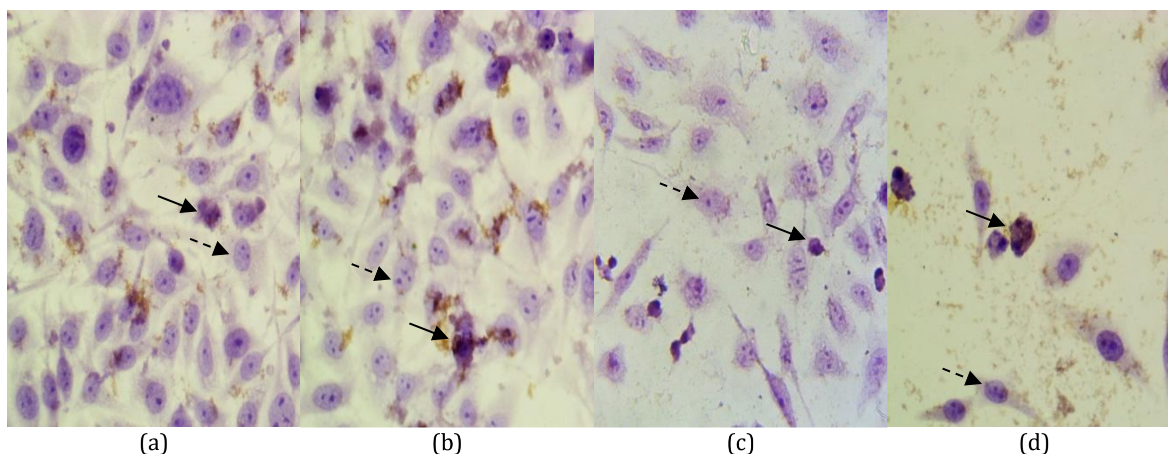
Immunocytochemistry Study

Regarding the color reaction in cells, the darker the color, the more cells express TNF alpha. The TNF-α gene expression was determined using the immunocytochemistry method with the principle of specific antibody binding (Nurani, 2011). Table 4 shows the TNF-α gene expression in T47D cells of Cisplatin as well as a combination of HS SNEDDS and Cisplatin. The HS SNEDDS and Cisplatin combination exhibited a significantly ($p<0.05$) higher TNF-α gene expression than other samples. Moreover, Figure 2 indicates that the different brown colors produced in the cytoplasm appear in the immunocytochemistry picture. Figure 2(d) exhibits more dark brown cells than the others, showing the highest TNF-α gene expression (Mohamad, 2016).

Table 4. TNF-α gene expression of various samples and control in T47D Cells. Data are presented as mean ± standard deviation (n = 3)

Groups	% TNF-α Gene Expression
Cisplatin 6.269 µg/mL	12.724 ± 4.916
HS SNEDDS 241.99 µg/mL	10.334 ± 2.943
HS SNEEDS 120.995 µg/mL + Cisplatin 3.135 µg/mL	17.939 ± 7.323*
Control	8.791 ± 2.896

Description = *significantly different from the control group ($p<0.05$).



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

Figure 2. Immunocytochemistry picture of TNF-α Expression in T47D Cells of (a) Control, (b) HS SNEDDS, (c) Cisplatin, (d) HS SNEDDS+Cisplatin. Microscopic visualization at a magnification of 40x. (-->: Do not express TNF-α , → : Expressing TNF-α).

Based on the results obtained, it is evident that the combination of HS SNEDDS and Cisplatin can increase TNF-α gene expression compared to control and single administration. The mechanism is via apoptosis through the extrinsic pathway by increasing TNF-α gene expression. The interaction of TNF-α with cancer cells can cause activation of proapoptotic caspases, essential enzymes in the apoptosis process, thereby promoting cell death (Diaz Arguello and Haisma, 2021). In addition, it can be seen in Figure 2 that the

combination of HS SNEDDS and Cisplatin groups indicates the decrease in the dark brown cells, proving that this combination can increase cancer cell death. Therefore, HS SNEDDS can be used as a Cisplatin co-chemotherapy agent to improve its effect.

CONCLUSIONS

HS SNEDDS exhibits antiproliferative properties against T47D cells by delaying cell doubling time, as indicated by a higher doubling time compared to the control. The combination of HS SNEDDS and Cisplatin, with the combination of HS SNEDDS and Cisplatin by 30.249 and 6.269 μg/mL, respectively, provided the strongest synergism of Combination Index (CI) by 0.17. Furthermore, combining HS SNEDDS and Cisplatin could significantly increase TNF-α gene expression compared to the control and single treatment through apoptosis with the extrinsic pathway. Therefore, HS SNEDDS can be potentially used as a co-chemotherapy agent for Cisplatin, leading to an improvement in the anticancer activity of Cisplatin.

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CONFLICT OF INTEREST

There is no conflict of interest.

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