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Development of Self-Nano Emulsifying Drug Delivery System (SNEEDS) Containing *Hibiscus sabdariffa* L. Extract, an Anticancer against T47D Cells, as a Co-Chemotherapy of Cisplatin

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Abstract: The first-line chemotherapy drug for breast cancer is cisplatin. However, it shows a high incidence of resistance. Therefore, *Hibiscus Sabdariffa* L (HS), containing strong anticancer compounds, was developed using a self-nano-emulsifying drug delivery system (SNEDDS), potentially as cisplatin co-chemotherapy. This study aimed to develop the HS SNEDDS providing anticancer activity against T47D breast cancer cells. The methods consist of the development of HS SNEDDS (F1-F7) with various surfactant and co-surfactant concentrations, followed by characterization of HS SNEDDS. The selected formulation was evaluated regarding the cytotoxicity on T47D cells and selectivity on Vero cells. The results showed that F7, as a selected formulation, indicated a transmittance, globule size, PI, zeta potential, and emulsification time of 97.80%, 15.68 ± 0.19 nm, 0.12 ± 0.01, -8.05 ± 1.88 mV, and 24.76 ± 0.29 seconds, respectively. Furthermore, according to the data of toxicity and selectivity studies, HS SNEDDS was proven to enhance the toxic properties of HS extract on T47D cells and be safe on normal cells. In conclusion, HS SNEDDS providing the required characteristics could be obtained, and potentially be used as a co-chemotherapy of cisplatin, showing the cytotoxic effect on T47D breast cancer cells.

Keywords: anticancer, *Hibiscus sabdariffa*, SNEDDS, T47D

1. INTRODUCTION

The incidence of breast cancer is high, with about 2.3 million new cases and almost 700,000 deaths due to this disease in 2020. It is 11,6% of all cancer types for women in the world [1], [2]. The first chemotherapeutic for breast cancer is Cisplatin; however, it indicates a high toxicity and drug resistance due to the high dose of therapy [3], [4]. Therefore, a co-chemotherapeutic is needed to improve the therapeutic effect of cisplatin for treating breast cancer.

Hibiscus sabdariffa L. is a natural co-chemotherapeutic providing several pharmacological activities, including immunomodulating, antioxidant, and cytotoxic effects against breast cancer. *Hibiscus Sabdariffa* L contains various substances, including anthocyanin (flavonoid), quercetin, gossytrin, sabdaritrin, gossypitrin, gossypetin glucoside, luteolin, steroid, triterpenoid, saponin,

tannin, and β-carotene [5]. The anthocyanin contained in *Hibiscus Sabdariffa* L. provides antioxidant activity that is higher than α-tocopherol (vitamin E), ascorbic acid, and beta-carotene [6]. In addition, anthocyanins can inhibit tumor invasion and metastases and act as a mediator of angiogenesis in cancer therapy by modifying several receptors [7]. Edityaningrum et al. (2024) concluded that an ethanolic extract of Hibiscus Sabdariffa L effectively inhibited T47D cells (IC_{50} = 32.3±2.15 μg/mL). In Addition, a previous study showed the safety of an ethanolic extract of *Hibiscus Sabdariffa* L. on organ vital signs, including the kidney and liver [8].

However, its oral bioavailability is low (<2%), possibly due to limited phase I and II metabolites, metabolites produced by microbes, and conjugation products [9]. Another limitation is related to the stability of anthocyanins, which is influenced by several factors such as light, oxygen, enzymes, and pH, especially in the neutral and alkaline environment [10]. Therefore, a promising strategy to overcome this issue was developed, a nano-delivery system for anthocyanin by incorporating it into a carrier, namely, a self-nano-emulsifying drug delivery system (SNEDDS).

SNEDDS is a lipid-based nanocarrier system containing oil, surfactant, and cosolvent or cosurfactant. SNEDDS offer many advantages, including enhancing drug solubility as well as stability and preventing first-pass metabolism, and drug degradation against pH and enzyme presence in the gastrointestinal tract (GIT) [11]. Previous studies showed that SNEDDS was applied as a carrier of flavonoids derived from wheat [12] and Naringenin from Bali orange [13]. Additionally, a previous study showed that combining *Citrus aurantium* L. and *Rose damascene flowers* improved cytotoxicity after incorporation in the SNEDDS [14]. Another study proved that using SNEEDS as a carrier could enhance the anticancer activity of capsaicin through apoptosis in HT-29 colorectal cancer cells. SNEDDS containing capsaicin at a 100 μg/mL concentration indicated the inhibition of cell viability and apoptosis ability of 67.70% and 35.84%, respectively. In contrast, capsaicin without a carrier exhibited inhibition of cell viability and apoptosis ability of 43.93% and 14.06%, respectively [15].

Therefore, this study aimed to develop SNEEDS formulation as a nanocarrier for *Hibiscus Sabdariffa* L (HS), providing anticancer activity against T47D breast cancer cells and potentially be used as co-chemotherapy with cisplatin. T47D breast cancer cells were selected in this study due to their sensitivity to progesterone compared to the MCF-7 cell line [16].

In this study, an HS SNEDDS formulation was developed by varying the concentration of Tween 80 as a surfactant and PEG 400 as a co-surfactant. The optimum formulation was obtained by measuring the characteristics of HS SNEDDS, including transmittance, globule size, polydispersity index (PI), zeta potential, and emulsification time. Furthermore, the optimum formulation was performed regarding the cytotoxicity on T47D breast cancer cells and selectivity on Vero cells.

2. MATERIALS AND METHODS

Hibiscus Sabdariffa L flower was supplied from Merapi Pharma Herbal, Yogyakarta. Fish cucut oil (Centrophorus sp.) was obtained from PT Bumi Wijaya, Cilacap. Tween 80 and PEG 400 were purchased from PT Brataco. T47D and Vero cells were obtained from an in vitro cell culture laboratory (Parasitology Laboratory, Medical Faculty, Gadjah Mada University). T47D and Vero cells were stored in RPMI and DMEM media. The storage condition was completed with fetal bovine serum (FBS) with 5% CO_2 at 37°C. Cisplatin was purchased from PT Kalbe Farma, Jakarta.

2.1. Extraction of Hibiscus sabdariffa (HS)

The *Hibiscus sabdariffa* (HS) flower petals were dried in the oven at a temperature of less than 60°C for 24 hours, followed by grinding to obtain powder, and then sieved using a 40-mesh sieve. Subsequently, 700 g of powder was extracted using the maceration method with one liter of 96% ethanol, and the stirring was carried out for one hour, followed by keeping at room temperature for 24 hours. Remaceration was conducted 2-fold using the same solvent. The produced extract was filtered using a paper filter, and the filtrate was evaporated using a rotary evaporator and water bath at 50°C to obtain the viscous extract.

2.2. Preparation of HS SNEDDS

HS SNEDDS was prepared based on the formulation listed in Table 1. Briefly, the HS extract was dissolved in the cucut oil by stirring using a magnetic stirrer (Thermoline) at room temperature for 15 minutes (A mixture). The B mixture was prepared by mixing the Tween 80 and PEG 400 using a magnetic stirrer at room temperature for 10 minutes. Afterward, the A and B mixture was homogenized using a probe sonicator (Biobase UCD-250) with an intensity of 40% for 10 minutes [17].

Table 1. Formulation of HS SNEDDS with various concentrations of Tween 80 as a surfactant and PEG 400 as a co-surfactant

Formulation	HS extract (% b/b)	Cucut fish oil (%b/b)	Tween 80 (% b/b)	PEG 40 (%b/b)
1	10	10	55	25
2	10	10	52	28
3	10	10	50	30
4	10	10	58	22
5	10	10	45	35
6	10	10	60	20
7	10	10	65	15

2.3. Characterization of HS SNEEDS

The characteristics of HS SNEDDS, including transmittance, globule size, polydispersity index, zeta potential, and emulsification time.

2.3.1. Measurement of transmittance

The transmittance measurement was carried out by dispersing 100 µL of HS SNEDDS in the 10 mL Artificial Intestinal Fluid (AIF) PH 7,2 using a magnetic stirrer at 37 °C and a speed of 300 rpm for 1 hour. Subsequently, the dispersed sample was determined the transmittance using a Spectrophotometer UV-VIS (Shimadzu UV-1900i) at 650 nm.

2.3.2. Measurement of globule size, polydispersity index (PI), and zeta potential

Briefly, 100 µL of HS SNEDDS was dispersed in the 10 mL Artificial Intestinal Fluid (AIF) PH 7,2 using a magnetic stirrer at 37°C and a speed of 300 rpm for 1 hour. Subsequently, the dispersed sample was measured for the globule size, polydispersity index, and zeta potential using a zeta sizer (Malvern).

2.3.3. Measurement of emulsification time

Briefly, 1 mL of HS SNEDDS was dropped into 100 mL of Artificial Gastric Fluid (AGF) pH 1-2, followed by stirring using a magnetic stirrer at 120 rpm at 37°C. Afterward, the time required to disperse the sample was observed and recorded as the emulsification time.

2.3.4. Physical storage stability study

This study was conducted against the selected formulation to ensure no change in the HS SNEDDS composition properties affecting its characteristics. The experiment was conducted by storing the HS SNEDDS at room temperature for 30 days and observing the properties on days 0, 15, and 30, including the globule size, polydispersity index, and zeta potential.Rosela

2.4. Toxicity studies of HS SNEDDS on T47D cells and selectivity studies on Vero cells

The cytotoxicity and selectivity studies were performed using cell lines including T47D and Vero cells. The experiment was conducted by incubating the samples in a 96-well microplate with a 2 × 104/ well density in the maintenance condition of 5% CO2 concentration and a temperature of 37°C. Every well was filled with the HS extract and HS SNEDDS by 31, 25, 62.5, 125, 250, and 500 μg/mL, whereas the cisplatin was prepared with the concentrations of 1.56, 3.13, 6.25, 12.50, and 25 μg/mL Subsequently, 100 μL of 0.5% MTT in phosphate buffer Saline (PBS) was added followed by incubating for 4 hours. Afterward, each well was added with 10% sodium dodecyl sulfate (SDS) and then incubated for 24 hours at room temperature in dark conditions by covering it with aluminum foil. To determine the cytotoxicity and IC50, the analyses using an ELISA reader (Biorad, Inggris) at 595 nm were carried out by measuring the colour intensity [18]. The following equations, 1 and 2, were used to calculate the cytotoxicity and selectivity, respectively. A linear regression correlating percent viability as a function of log drug concentration was constructed to calculate IC50, a concentration resulting in 50 percent cell viability.

$$\frac{\text{(Absorbance treatment-Media control absorbance)}}{\text{(Cell control absorbance-Media control absorbance)}} \times 100\% \quad \text{..... (Equation 1)}$$

$$SI = \frac{\text{IC50 Vero Cell}}{\text{IC50 T47D Cell}} \times 100\% \quad \text{..... (Equation 2)}$$

2.5. Data Analysis

A statistical study was performed using the One-Way ANOVA (IBM SPSS Statistics 23) followed by post-hoc Tukey, to evaluate the HS SNEDDS formulation optimization by comparing the characteristics of each formulation. In addition, the same statistical analysis was also used to compare the treatments and control groups in the cytotoxicity study.

3. RESULTS AND DISCUSSION

3.1. Preparation and characterization of HS SNEEDS

HS SNEDDS was prepared with various concentrations of surfactant and co-surfactant, namely, Tween 80 and PEG 400, respectively. The resulting formulations are shown in Figure 1, indicating that all formulations (F1–F7) provide a similar visual appearance, including the dark brown color, homogeneity, and no precipitate particles. It is assumed that the HS extract could be incorporated into SNEDDS as a carrier. However, each formulation indicated different characteristics, including the transmittance, globule size, polydispersity index (PI), zeta potential, and emulsification time. Based on Table 2. Formulation 7 (F7) exhibits better characteristics than others, showing the highest transmittance of 97.80%, the lowest globule size, PI, and emulsification time of 15.68 ± 0.19 nm, 0.12 ± 0.01, and 24.76 ± 0.29 seconds, respectively. Therefore, based on those properties of F7, this formulation was selected as a better SNEDDS formulation. The possible reason is that F7 contains the highest surfactant concentration, namely, Tween 80, as shown in Table 1. The higher surfactant

concentration leads to a smaller globule size being produced. Previous studies also exhibited that the surfactant concentration impacts the particle or globule size. For instance, in the formulation of curcumin-loaded nano-emulsion, the higher the surfactant concentration, the smaller the particle size produced. In addition, after storage for 15 days, the nano-emulsion systems were stable, and there were no aggregations [19]. Moreover, the particle size affects the ability to pass through the membrane; the smaller the globule or particle size, the higher the nanocarrier's possibility to permeate the intestinal mucosal layer, leading to the ease of reaching the intestinal membrane, and thus entering into the systemic circulation [20], [21], [22], [23], [24].

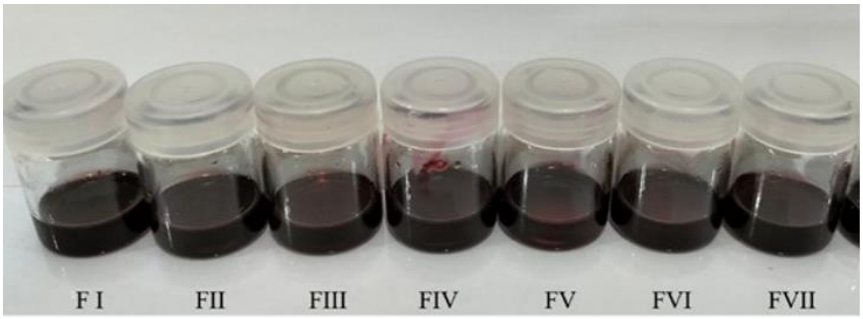

Figure 1. HS SNEDDS formulation optimization with various concentrations of Tween 80 and PEG 400 as surfactant and co-surfactant.

Another property, such as transmittance, can also be used as a parameter of the nanocarrier quality. The transmittance percentage, which is closer to 100%, is beneficial since it can describe the transparency of the systems. The higher the value of the transmittance percentage, the more transparent the systems and the smaller the particle size produced. An additional important characteristic is a PI describing the particle distribution in the nanocarrier system, thereby influencing the stability and homogeneity of colloidal systems; the lower the PI, the closer to zero, the more stable the nanocarrier [25], [26]. Zeta potential is another property of the system's stability and ability to transport or permeate through the membrane. Based on data in Table 2, all formulations show a negative zeta potential, but it is not too negative.

Table 2. Characterization of HS SNEDDS, including transmittance, globule size, polydispersity index, zeta potential, and emulsification time. Data are presented as mean ± SD (n = 3)

Formulation	Transmittance (%)	Globule size (nm)	*Polydispersity Index (PI)*	Zeta Potential (mV)	Emulsification time (seconds)
1	96.58 ± 0.08	20.1 ± 7.30	0.3690 ± 0.08	-2.833 ± 0.561	41.76 ± 1.568
2	95.64 ± 0.04	18.19 ± 0.27	0.2132 ± 0.02	-8.64 ± 2.184	26.90 ± 1.161
3	96.44 ± 0.04	19.36 ± 0.28	0.2964 ± 0.03	-6.014 ± 0.256	36.87 ± 1.443
4	75.23 ± 0.05	19.58 ± 0.31	0.2858 ± 0.04	-6.792 ± 3.993	27.70 ± 1.360
5	86.33 ± 0.03	19.7 ± 0.30	0.2861 ± 0.02	-2.009 ± 1.050	27.74 ± 0.636
6	97.25 ± 0.03	18.28 ± 1.21	0.2707 ± 0.06	-5.104 ± 1.276	28.98 ± 0.915
7	97.83 ± 0.05	15.68 ± 0.19	0.1224 ± 0.01	-8.047 ± 1.878	24.76 ± 0.296

In the case of SNEDDS, the zeta potential value impacts the tendency of globule aggregation during administration, after contact with the gastrointestinal fluid, resulting in an impact on the capability of the nanocarrier to pass through the membrane. A less negative or positive zeta potential value of SNEDDS globules, close to zero, leads to a higher tendency to form aggregates. During the storage of SNEDDS, there may be a change in the SNEDDS preconcentrate properties, influencing the SNEDDS characteristics.

Furthermore, to confirm that SNEDDS can be dispersed immediately in the gastrointestinal fluid, the emulsification time study was carried out by dispersing the HS SNEDDS in the simulated gastrointestinal fluid medium with a pH of 1-2. The fast emulsification time of SNEDDS means that the SNEDDS can be dispersed well in the medium. F7 indicates the highest emulsification time compared to other formulations, as shown in Table 2. The ease of being dispersed in the medium can be affected by the surfactant concentration due to the ability of the surfactant to emulsify the SNEDDS droplet in the medium to form the oil-in-water (O/W) emulsion type. In addition, the surfactant can also maintain the nanocarrier stability by preventing the coagulation of SNEDDS globules.

Moreover, it is necessary to ensure that the HS SNEDDS formulation is stable during storage. F7, as a selected formulation, was conducted for stability study by storing at room temperature for 30 days, for observation on the 0, 15, and 30 days regarding the changing of the properties of HS SNEDDS composition, potentially affecting the physical characteristics of HS SNEDDS once dispersed. Based on Table 3, during 30 days of storage, fewer changes are not significantly different ($P>0.05\%$) regarding the characteristics, including globule size, PI, and zeta potential. It can be concluded that the HS SNEDDS was stable during storage.

Table 3. Stability study of F7 by storing the SNEDDS at room temperature for 30 days. Data are presented as mean ± SD (n = 3)

Characteristics of HS SNEDDS	Days		
	0	**15**	**30**
Globule size (nm)	15.68 ± 0.19	17.37 ± 0.85	17.89 ± 0.52
PI	0.12 ± 0.01	0.15 ± 0.03	0.19 ± 0.01
Zeta potential (mV)	-8.05 ± 1.88	-7.29 ± 0.74	-5.76 ± 1.32

3.2. Toxicity and selectivity studies of HS SNEDDS

Cytotoxicity study of HS extract, HS SNEDDS, and Cisplatin on T47D and Vero cells was conducted to determine IC_{50} using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. IC_{50} is a drug concentration required to kill 50% of cells; the lower IC50 value indicates a higher drug effectiveness [27]. The MTT study was carried out based on the conversion of yellow tetrazolium salt to a coloured formazan product by mitochondrial enzymes from cells, followed by measuring using an ELISA Reader, and the absorbance obtained was correlated with the number of living cells [28]. Figure 2 exhibits the cell viability of T47D and Vero cells incubated in various concentrations of HS-SNEDDS. The cytotoxicity of HS SNEDDS on the T47D cells was higher than on Vero cells (normal cells), indicated by up to a sample concentration of 500 μg/mL; the viability of Vero cells was still more than 60%, whereas the viability of T47D cells dropped to less than 20%.

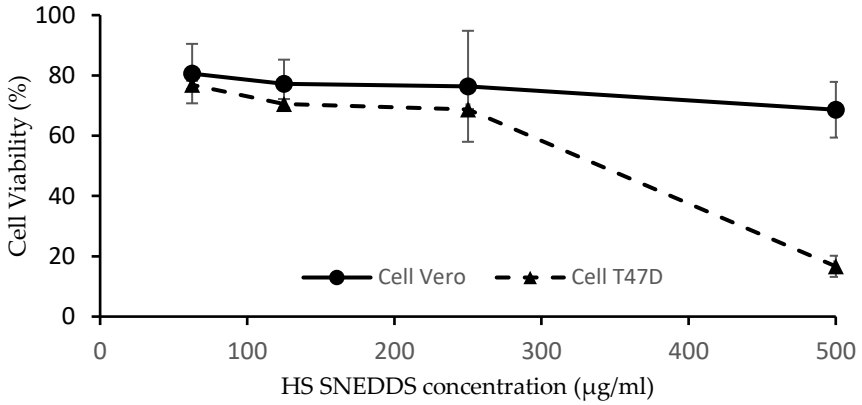

Figure 2. Viability of T47D (striped line) and Vero (solid line) cells after incubation in various HS SNEDDS concentrations. Data are presented as mean ± SD (n = 3)

Regarding the IC_{50}, a compound is categorized as toxic, moderate, or non-toxic if the IC50 value is less than 100 µg/mL, 100-1000 µg/mL, or more than 1000 µg/mL, respectively [29]. Based on the results in Table 4, it can be concluded that the HS and HS SNEDDS extracts are quite toxic to the cells, whereas cisplatin is more toxic. HS SNEDDS was proven significantly ($P<0.05$) to increase the toxicity properties of HS extract, with a smaller IC_{50} of HS SNEDDS compared to HS extract.

Table 4. IC50 of samples, including HS extract, HS SNEDDS, and cisplatin on T47D cells. Data are presented as mean ± SD (n = 3)

Samples	IC50 (mg/mL)
HS extract	0.37 ± 0.02
HS SNEDDS	0.24 ± 0.02
Cisplatin	0.01 ± 0.001

Table 5. Selectivity index (SI) of HS SNEDDS and Cisplatin. Data are presented as mean ± SD (n = 3)

Samples	IC50 Vero (mg/mL)	IC50 T47D (mg/mL)	SI
HS SNEDDS	30302.08 ± 6.41	0.24 ± 0.02	108101.25
Cisplatin	52.36 ± 24.47	0.01 ± 0.001	8351.84

Furthermore, to describe the ability of a drug or carrier to achieve the specific cell target, the Selectivity Index (SI) of HS SNEDDS and Cisplatin was determined. A compound is said to have high or low selectivity if the SI value is greater or less than 3, respectively [30]. Anticancer drugs that provide a specific target to cancer cells rather than healthy cells are correlated with increased efficacy and reduced side effects [31]. Hibiscus Sabdariffa L extracts (methanolic and water) exhibit high selectivity for cancer cell targets, alongside 6.1 and 5.2, respectively (Torky and Hossain, 2017). Table 5 indicates that the SI of HS SNEDDS is significantly ($P<0.05$) higher than that of Cisplatin. HS contains flavonoids, especially anthocyanins, gossypetin, and hibiscetin, as potential compounds exhibiting anticancer properties due to their antioxidant activity by combating oxidative stress associated with cancer development [32], [33]. In addition, HS anticancer activity also occurs through various mechanisms, such as triggering programmed cell death in cancer cells [34], inducing G2/M phase arrest, preventing cancer cell proliferation [35] and inhibiting cancer cell growth through various signaling pathways [36]. Ahirwar and Ahirwar (2020) described a ROS-mediated pathway

for delaying cancer cell growth, implying that the phenolic compounds in this herb play an important role in this mechanism. The results of the cytotoxic studies on T47D and Vero cells above indicate that HS SNEDDS could increase the toxicity effect of HS extract on T47D breast cancer cells, and was not toxic to Vero cells like normal cells. Therefore, HS SNEDDS can potentially be used as a co-chemotherapy of Cisplatin.

4. CONCLUSION

In this study, an HS SNEDDS formulation could be developed, showing the characteristics of F7 as a selected formulation that meets the requirements, including transmittance, globule size, polydispersity index (PI), zeta potential, and emulsification time. Based on the data of toxicity and selectivity studies, HS SNEDDS was proven to increase the toxic properties of HS extract on T47D cells and be safe on normal cells. Therefore, HS SNEDDS can potentially be used as a co-chemotherapy of Cisplatin to enhance its action.

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