Optimization of Self-Nanoemulsifying Drug Delivery System (SNEDDS) of *Annona muricata* L. leaves chloroform extract using VCO (Virgin Coconut Oil) as an oil phase

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**Abstract.** *Annona muricata* L. also called as soursop is one of medicinal plants that have a broad spectrum of biological activities and the most impressive of which is anticancer activity. Annonaceous acetogenins (ACGs) have been shown to successfully induce death in cancer cell. However, the solubility of ACGs is poor, so modification of formula of Self-Nanoemulsifying Drug Delivery System (SNEDDS) can be used for improving drug solubility. This study was used *Annona muricata* L. leaf chloroform extract then optimized to formula of SNEDDS using D-Optimal mixture design (Design Expert 9 Trial software). Sixteen formulas from the software consisting of oil phase (VCO), surfactant (tween 80-cremophor EL), and co-surfactant (propylene glycol) were tested of loading dose extract and characterized for transmittance, emulsification time, and phase separation. One sample t-test was used for verification, then the optimum SNEDDS was evaluated against particle size, polidispersity index, zeta potential, and TLC test was used to predict the active ingredient in the extract. The optimum SNEDDS consisting of 22.00% oil : 60.79% surfactant : 17.21% co-surfactant showed desirability value 0.608 and can loaded 25 mg/g extract. The optimum SNEDDS showed 84.4% (SE ± 1.64) of transmittance; 14.79 seconds (SE ± 1.81) of emulsification time; and 0.898 (SE ± 0.01) of phase separation. The verification test showed no significant difference (p >0.05) between the observation and the prediction from the software. Evaluation results showed 42.73 nm (SE ± 0.49) of the particle size; polidispersity index 0.37 (SE ± 0.10); -27.43 mV (SE ± 0.26) of zeta potential, and TLC results showed similarity of active ingredient in the extract with the active compound of the fraction. Therefore, from these results suggest that the optimum SNEDDS achieved the nanoparticle size for SNEDDS formulation and may be improving drug solubility.

1. **Introduction**

*Annona muricata* L. also called as soursop is one of medicinal plants that have a broad spectrum of biological activities and the most impressive of which is anticancer activity. Annonaceous acetogenins (ACGs) have been shown to successfully induce death in cancer cell [1]. Besides that, the other compounds such as flavonoids also play an important role in induce death in cancer cell. According to Yang et al [2] inhibition of prostate tumor growth increased by synergistic interactions among ACGs and flavonoids in soursop leaves extract. Phytochemical study of soursop leaves extract that has been done shows that the level of flavonoids significantly higher concentration in chloroform extract than methanolic extract [3]. Soursop leaves also rich of lipid content compared to the other part of the plant due to the presence of ACGs which are derivates of long chain fatty acids (C32 or C34) [4]. But, ACGs are highly insoluble in water (<1 µL/mL) so their drug formulation have been limited [5].
However, these poorly water soluble drugs leads to low oral bioavailability. Yang et al [2] showed that the maximum plasma concentration of oral administration of 100 mg/kg soursop leaves ethanolic extract was less than 40 ng/mL in <1.0 hour [2]. Tablet dosage formulation of the soursop ethanolic leaves extract also has poor solubility when the concentration were tested [6]. Self-Nanoemulsifying Drug Delivery System (SNEDDS) is one of the lipid based drug delivery system that efficient for improving drug solubility. Hence, this study was aimed to optimized *Annona muricata* L. leaves chloroform extract to formula of SNEDDS that consist of oil phase (VCO), surfactant (tween 80-cremophor EL), and co-surfactant (propylene glycol) to achieve physical stability and further enhance drug solubility.

2. Experimental

2.1. Materials
Soursop fresh leaves (Karanganyar, Central Java), aquabides (IKA), analytical grade solvents like chloroform, methanol, ethyl acetate, and hexane, TLC silica gel 60 F254 (Merck), AGF, cremophor EL (Kolliphor® EL Sigma pH 6,0-8,0), tween 80, propylene glycol, glycerin, VCO, PKO, olive oil, aquades (Brataco). Oven, blender (Philips), analytical balance (Mettler Toledo), rotary evaporator (Stuart), waterbath (Grant), vortex mixer (Maxi Mix), sonicator (Branson), magnetic stirrer (IKa), particle size analyzer (HORIZA SZ-100), micropipette (Gilson), pH meter (Eutech), centrifuge (Mini Spin plus), UV/Vis spectrophotometer (Genesys™).

2.2. Method
2.2.1. Preparation of Soursop Leaves Chloroform Extract
Fresh leaves were picked from the tree at Karanganyar, Central Java and identified in Biology Department, Faculty of Mathematics and Natural Sciences, Sebelas Maret University based on C.A. Backer & R.C. Bakhuisen van den Brink [7]. The leaves were separated from the stalk, washed, air-dried at room temperature (25 °C), then dried with oven at 45 °C, and crushed into coarse powder. Extract was prepared by percolating the dry powdered sample with chloroform for 2x24 hours. The liquid extract was thereafter concentrated using rotary evaporator and waterbath until giving some dark-green crude extract [8].

2.2.2. Solubility Study of The Extract in Oil Phase, Surfactant, and Co-Surfactant
A total of 1.0 mg extract was added into each eppendorf tube with 1.0 mL different component: oil (VCO, PKO, olive oil), surfactant (tween 80 (100%), cremophor EL (100%), Tween 80:cremophor EL (85%:15%)), and co-surfactant (propylene glycol, glycerin). The mixture was vortexed on vortex mixer for 1.0 minute, incubated in waterbath at 45 °C for 10 minutes, sonicated for 15 minutes, and then left for two days at room temperature. The samples were then centrifuged at 3000 rpm for 20 minutes to separate the undissolved extract. Component that was able to dissolve more of the extract were selected visually for optimization [9].

2.2.3. Optimization SNEDDS Formula with D-Optimal Mixture Design
Three independent variables were studied: oil (A), surfactant (B), and co-surfactant (C) against three response variables: transmittance (%), emulsification time (second), and phase separation (F) using D-Optimal mixture design. Oil, surfactant, and co-surfactant that showing maximum solubility of the extract from the previous study were chosen as independent variables. Table 1 showed the independent variables and the levels selected for optimization.

| Code | Selected Component | Low   | High  |
|------|--------------------|-------|-------|
| A    | Oil                | 12.00%| 22.00%|
| B    | Surfactant         | 60.00%| 70.00%|
| C    | Co-Surfactant      | 12.00%| 22.00%|
2.2.4. Drug Loading Measurement
Samples of soursop leaves chloroform extract (100, 50, and 25 mg) were added into 1.0 g SNEDDS system. SNEDDS were then homogenized with a vortex for 1.0 minute, sonication for 10 minutes, and incubation at 45 °C for 15 minutes in waterbath. The extract that was able to dissolve more in that system and did not show any signs of instability were selected visually for the drug loading dose per 1.0 g SNEDDS [10].

2.2.5. Characterization of SNEDDS
2.2.5.1. Transmittance
A 1.0 mL SNEDDS was added with aquabides until volume of 50 mL. The mixture was homogenized with a vortex for 1.0 minute. Percentage of transmittance then was analyzed by UV-Vis spectrophotometer at 650 nm [11].

2.2.5.2. Emulsification Time
A 200 µL SNEDDS was dripped quickly into 50 mL of Artificial Gastric Fluid (AGF) medium pH 1.2 at 37±0.5 °C under 100 rpm continuous stirring. The emulsification time was taken as the time to form homogenous mixture spontaneously upon dilution [12].

2.2.5.3. Phase Separation
SNEDDS was added into eppendorf tube 1.5 mL then was centrifuged at 3500 rpm for 3x10 minutes [13]. The phase separation was visually observed by comparing the height of emulsion that remain stable with the total height of emulsion [14].

2.2.6. Determination of Optimum Formula of SNEDDS and Verification
D-Optimal mixture design was used to determine the optimum formula of SNEDDS. Three replicates of these optimum formula of SNEDDS were characterized for transmittance, emulsification time, and phase separation and thereafter were verified using one sample t-test.

2.2.7. Evaluation of Optimum Formula of SNEDDS
2.2.7.1. Particle Size and Zeta Potential
A 1.0 mL SNEDDS was diluted with aquabides until volume of 50 mL, then homogenized with a vortex for 1.0 minute, and 3.0 mL of sample was evaluated against particle size, polidispersity index, and zeta potential using particle size analyzer (Nanopartica SZ-100, HORIBA).

2.2.7.2. Thin Layer Chromatography (TLC) Test
Chloroform extract (E) that used in this study and active fraction (F) of soursop leaves from the previous study [15] were developed in TLC plate with the mobile phase chlorofom:ethyl acetate (7:3 v/v). Then, the spots were visualized by UV lamp at 254 and 366 nm [16].

2.2.8. Data Analysis
Design Expert 9 Trial (Stat-Ease Inc.) software was used to determined the optimum formula of SNEDDS. The effect of oil, surfactant, and co-surfactant were evaluated against the respond and statistically significant result if p <0.05. Verification between predicted value and observation value was conducted using one sample t-test by analytical software and statistically no significant difference if p >0.05.

3. Result and Discussion
3.1. Characterization of Soursop Leaves Chloroform Extract
Determination result (113/UN27.9.6.4/Lab/2017) showed that the sample used in this study was from family Annonaceae, genus Annona, and species Annona muricata L. Characteristics of soursop extract correspond to the study from Shofa [17] that the extract have dark-green colored, high consistency,
distinctive smell of leaves, and moisture content was 1.3%. This moisture content result can illustrate the residual solvent and water content of the extract. The amounts of chloroform solvent residue of the pharmaceutical product should be less than 0.0006% [18]. But, these moisture content result in this study not yet fully illustrated the chloroform solvent residue because the water and volatile oil content also affect.

3.2. Solubility Study of The Extract in Oil Phase, Surfactant, and Co-Surfactant
Selection of right component is important prerequisite for formulation of stable SNEDDS. The drug should have good solubility in each component so as the precipitation of drug during shelf life of formulation and after dilution in GI lumen can be avoided [19]. The solubility result of the extract was maximum in VCO, tween 80:cremophor EL (85%:15%), and propylene glycol. So, these were selected component for further formulation of SNEDDS.

The predominant fatty acid in VCO is lauric acid (>47%) which is medium chain fatty acid (MCFA) [20]. High percentage of MCFA in oil phase have been preferred for nanoemulsion formulation to achieve stability [21]. Saryanti [22] that surfactant combination of tween 80:cremophor EL (85%:15%) enhance SNEDDS formulation stability. In addition, long chain alcohols (C8-C12) such as propylene glycol are good co-surfactant for SNEDDS formulation [23].

3.3. Formula Composition of SNEDDS using D-Optimal Mixture Design
D-Optimal mixture design of Design Expert 9 Trial software showed sixteen formulas with various concentration of oil, surfactant, and co-surfactant with the total concentration is 100% (Tabel 2). These 11 formulas from the software have different composition with 5 formulas replicated. This is in order to reduce any possible errors that may occur.

| Run | Percentage of Oil (A), Surfactant (B), and Co-Surfactant (C) | Characteristics of SNEDDS of Soursop Leaves Chloroform Extract |
|-----|-------------------------------------------------------------|---------------------------------------------------------------|
|     | Oil (%) | Surfactant (%) | Co-Surfactant (%) | Transmittance (%) | Emulsification Time (second) | Phase Separation (F) |
| 1   | 17.33   | 65.33         | 17.33             | 83.00             | 15.00                        | 0.923               |
| 2   | 12.00   | 66.00         | 22.00             | 88.40             | 8.00                         | 0.969               |
| 3   | 22.00   | 63.00         | 15.00             | 91.30             | 24.00                        | 0.885               |
| 4   | 22.00   | 66.00         | 12.00             | 84.90             | 28.00                        | 0.885               |
| 5   | 18.00   | 70.00         | 12.00             | 81.90             | 43.00                        | 0.923               |
| 6   | 22.00   | 60.00         | 18.00             | 86.20             | 6.00                         | 0.885               |
| 7   | 12.00   | 70.00         | 18.00             | 78.70             | 26.00                        | 0.961               |
| 8   | 12.00   | 70.00         | 18.00             | 83.80             | 30.00                        | 0.969               |
| 9   | 18.00   | 60.00         | 22.00             | 85.50             | 18.00                        | 0.923               |
| 10  | 12.00   | 66.00         | 22.00             | 84.90             | 11.00                        | 0.969               |
| 11  | 19.67   | 65.67         | 14.67             | 79.70             | 9.00                         | 0.904               |
| 12  | 18.00   | 70.00         | 12.00             | 76.80             | 39.00                        | 0.923               |
| 13  | 22.00   | 60.00         | 18.00             | 90.60             | 14.00                        | 0.885               |
| 14  | 15.00   | 63.00         | 22.00             | 85.90             | 8.00                         | 0.942               |
| 15  | 18.00   | 60.00         | 22.00             | 81.80             | 10.00                        | 0.923               |
| 16  | 15.00   | 70.00         | 15.00             | 70.60             | 20.00                        | 0.942               |

SNEDDS in 4 g system

3.4. Drug Loading
Drug loading measurement was aimed to identifying the maximum concentration of the extract that can dissolve homogeneously in the SNEDDS system. The test result obtained from drug loading at 25 mg/g indicated that the SNEDDS system is capable of dissolving extract. The solubility testing
parameters were indicated by the clarity, homogeneity, and no precipitation in the SNEDDS system when visually observed.

3.5. Characteristics of SNEDDS

Characteristics of SNEDDS describing physical stability such as transmittance, emulsification time, and phase separation were used to determining the optimum formula in this study. Percentage transmittance was used to describe the clarity of SNEDDS system formed in aqueous media. Nanoemulsion have droplets with diameter on order of less than 100 nm, so nanoemulsion are often transparent in appearance as the droplet size is significantly smaller than the wavelength of visible light [24]. The transmittance value from the 16 run formulas ranged from 70.60% to 91.30% (Table 2).

Emulsification time is an important parameter to assess efficiency of SNEDDS to form nanoemulsion spontaneously in GI media under mild agitation. A perfect SNEDDS formulation will able to disperse completely and quickly which takes about less than 1.0 minute with a clear appearance [11]. This study showed that 16 formulas of SNEDDS have emulsification time less than 1.0 minute with a clear appearance (Table 2).

Besides that, nanoemulsion also a kinetically stable system that takes quite long time to separate into different phase. Accelerated stability testing with centrifugation method was aimed to shorten the observation time by stirring at 3500 rpm for 20 minutes could be said equivalent to gravity for ±1,0 year [13]. The centrifugation force exerts pressure to the SNEDDS system thus destroying the interface layer which causes the oil to separate. Phase separation value (F) was obtained from the separation level of SNEDDS system. The higher the remaining stable layer, the higher the phase separation value will be.

3.6. Optimum Formula of SNEDDS and Verification

The experimental mixture design technique is applied for optimization for independent varibles when response variables are dependent only on the proportions of the ingredients of mixture. D-Optimal mixture design is commonly used to reveal main effects and interaction effects between the independent variables against response variables. The interaction between independent variables against response variables in this study showed by polynomial equation from the Design Expert 9 Trial software (Table 3).

Table 3. Physical stability responses of SNEDDS based on mathematics model and ANOVA statistical analysis.

| Response             | Polynomial Equation | Mathematics Model | Lack of Fit [p >0.05] | p-value (ANOVA) [p <0.05] |
|----------------------|---------------------|-------------------|-----------------------|--------------------------|
| Transmittance (%)    | Y = 13.45 A + 0.34 B + 3.17 C – 0.14 AB – 0.29 AC + 0.01 BC | quadratic          | 0.3175                | 0.0197                   |
| Emulsification Time (second) | Y = 20.93 A + 6.12 B + 33.14 C – 0.43 AB – 0.37 AC – 0.64 BC | quadratic          | 0.0885                | 0.0031                   |
| Phase Separation (F) | Y = 1.94 A + 0.01 B + 0.02 C – 1.17 AB – 9.77 AC – 2.27 BC | quadratic          | 0.6690                | <0.0001                  |

All physical stability responses provide a quadratic model because the ANOVA statistical analysis have p <0.05 (Table 3). These result supported with Lack of Fit >0.05 which means there is no significant difference between the observation value with the prediction value from the model so that the quadratic model is precise. The polynomial equation of transmittance and emulsification time response showed that interaction between surfactant and co-surfactant in the mixture give the greatest influence in increasing percentage transmittance with the coefficient +0.01 and decreasing the emulsification time with the coefficient -0.064. This result correspond with the study from Saryanti [22] in which increased of surfactant combination of tween 80-cremophor EL and co-surfactant propylene glycol may increase trasmittance. Moreover, emulsification time is also mediated by the
interaction of surfactant and co-surfactant by form the interface layer between oil and water [12]. Co-
surfactant helps surfactant in reducing the interface tension between oil and water so that
emulsification occurs spontaneously.

![Contour plot diagram](image)

Figure 1. Contour plot diagram physical stability responses of SNEDDS: (a) transmittance, (b) emulsification time, and (c) phase separation. (d) Super impose diagram showed the optimum area of the oil : surfactant : co-surfactant component.

Contour plot diagram showed the interaction between oil (VCO), surfactant (tween 80- cremophor EL), and co-surfactant (propylene glycol) against each of the responses. The highest response indicated by the red area and the lower response indicated by the area of yellow, green, light blue, until dark blue (Figure 1). Based on optimization result, selected optimum formula of SNEDDS showed highest desirability value (0.608) in super impose diagram. This formula will produce optimum characteristic value that correspond with the desired value. The composition of the optimum formula is 22.00% oil : 60.79% surfactant : 17.21% co-surfactant.

Verification of optimum formula was aimed to compare between observation value with prediction value from the Design Expert 9 Trial software. Non-statistically significant results (p >0.05) between predicted and observed value of transmittance, emulsification time, and phase separation on one sample t-test were shown in Table 4. As the result, it can be concluded that the optimum formula suggested by Design Expert 9 Trial software have no significant difference with the observation value so fulfill the verification.
Table 4. Verification of optimum formula of SNEDDS.

| Response                        | Prediction | CI          | Observation ± SE | p-value [p >0.05] |
|---------------------------------|------------|-------------|------------------|-------------------|
| Transmittance (%)               | 88.39      | 84.09 - 92.71 | 84.64±1.64       | 0.150             |
| Emulsification Time (second)    | 12.91      | 20.60 - 7.19 | 14.79±1.81       | 0.409             |
| Phase Separation (F)            | 0.884      | 0.88 - 0.89  | 0.898±0.01       | 0.393             |

n=3 for each response

3.7. Evaluation of Optimum Formula of SNEDDS
3.7.1. Particle Size and Zeta Potential. Result of the particle measurement was 42.73 nm with polidispersity index (PI) value 0.37. The optimum SNEDDS showed uniform particle size distribution because the PI value closer to 0.0 [11]. Zeta potential was used to determine the value of repulsion between dispersion particles so the aggregation and phase separation can be avoided. The zeta potential in this research was high enough, that was -27.43 mV.

3.7.2. Thin Layer Chromatography (TLC). Active ingredient in the chloroform extract (E) was predicted using simple TLC method that use active fraction (F) of soursop leaves as positive control (Figure 2). These active fraction obtained from the previous study showed inhibition activity against HeLa cells with IC\textsubscript{50} 96.00 µg/mL [15]. TLC spot at UV\textsubscript{366 nm} with hRf value 95 was identified as active ingredient in comparison with active fraction that had the same hRf value. These result according to Pranatami [17] that hRf value of the most active fraction of soursop leaves was 95 with the same mobile phase in this study. Moreover, all of the plates showed a light-red alongside the spots. It is important to show that there was similarity of active ingredient in the extract with the active compound of the fraction.

![TLC profile](image)

**Figure 2.** TLC profile of chloroform extract (E) and active fraction (F) of soursop leaves visualized by (a) visible light, (b) UV\textsubscript{254 nm}, and (c) UV\textsubscript{366 nm}. The stationary phase was TLC silica gel 60 F\textsubscript{254} and the mobile phase was chloroform:ethyl acetate (7:3 v/v).

4. Conclusion
The optimum formula of SNEDDS of soursop leaves chloroform extract based on D-Optimal mixture design was 22.00% VCO, 60.79% tween 80-cremophor EL, and 17.21% propylene glycol with drug loading extract was 25 mg/g system. The verification test showed no significant difference (p >0.05) between the observation and the prediction from the software. The optimum formula of SNEDDS
fulfill the criteria of nanoemulsion with the 84.64% (SE ± 1.64) of transmittance; 14.79 seconds (SE ± 1.81) of emulsification time; and 0.898 (SE ± 0.01) of phase separation; 42.73 nm (SE ± 0.49) of the particle size; polidispersity index 0.37 (SE ± 0.10); and -27.43 mV (SE ± 0.26) of zeta potential. In addition, from the TLC results, we can predict that there was similarity of active ingredient in the extract with the active compound of the fraction.

Acknowledgement
This work is a collaboration with Natural Medicine Research Group and was supported by grant project from Sebelas Maret University.

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