

Cytotoxicity Effect of Self-Nanoemulsifying Drug Delivery System from Chloroform Extract of Bay Leaf (*Syzygium Polyanthum* (Wight) Walp.) with Oleic Acid as a Carrier

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Abstract

Bay leaves are used as food flavoring and also have medicinal properties. They may have cytotoxic effects derived from natural ingredients. The low efficacy of the therapy with an adequate dose preparation of the plant extract is due to its low solubility and oral bioavailability that is less than the maximum. Hence, this study aimed to improve the solubility and oral bioavailability of the extract mainly for the chloroform extract of leaves that are not soluble in water by preparing a self-nanoemulsifying drug delivery system (SNEDDS). Then, the potential cytotoxic effects of the SNEDDS of bay leaves were determined by calculating the value of IC_{50} on the T47D cell line. The cytotoxic effect of the SNEDDS of bay leaves was determined using an MTT assay, and the findings were read using an ELISA reader. Data analysis is calculated via linear regression methods by using Microsoft Excel software. The results showed that the SNEDDS of bay leaves performed cytotoxic effects on the T47D cell line with IC_{50} 138 μ g/mL. The results showed that the optimal composition formula SNEDDS, namely, Tween 20:PG:oleic acid = 2.25:2.25:0.5 in 5 mL SNEDDS preparation, which had a value of transmittance of 83.81% with emulsification time was less than 5 min; the average droplet size was 165.5 nm, and the zeta potential was -0.4 mV. The data analysis showed that the cytotoxicity effect of the SNEDDS of bay leaves is included in the moderate cytotoxic category.

Keywords: Bay leaf, optimization, nanoemulsi, cytotoxicity.

INTRODUCTION

Bay leaves are used as food flavoring and have medicinal properties. They can be used as antidiabetic agents since the compounds they contain, namely, eugenol, tannins, and flavonoids, are known to reduce blood sugar levels (Taufiqurrohman, 2015). Lajuck (2012) stated that flavonoid compounds, saponins, tannins, phenols,

and alkaloids in bay leaves can reduce low-density lipoprotein levels and increase high-density lipoprotein levels.

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The low efficacy of the therapy with adequate doses of plant extract preparation is caused by the low solubility and oral less-than-the-optimal bioavailability of the extract. Therefore, the preparation was formulated through a self-nanoemulsifying drug delivery system (SNEDDS) to increase the solubility and oral bioavailability of the extract, especially for the chloroform extract of bay leaves, which is insoluble in water. The SNEDDS is a system comprising a mixture of oil, surfactant, and cosurfactant that can form an oil-in-water nanoemulsion spontaneously when it encounters the aqueous phase through mild agitation in the stomach with the emulsion droplet size ranging from nanometers (Date, *et al.*, 2010).

The composition of the oil in the SNEDDS formula will determine the size of the nanoemulsion formed, the selection of the type of oil is based on its ability to dissolve the drug. Oil is the drug base in SNEDDS, and oleic acid was used in this study as a component of the oil. Due to its high self-emulsifying ability and significant drug dissolving capacity, oleic acid was chosen as the oil phase in the SNEDDS formulation (Kurakula & Miryala, 2013). The use of oleic acid in SNEDDS is expected to produce nanoemulsions to increase the oral bioavailability of bay leaf chloroform extract.

Surfactants play a role in lowering the surface tension. The choice of surfactant in SNEDDS is generally based on the safety of use and the value of the hydrophilic-lipophilic balance (HLB). Tween 20 and Tween 80 were chosen as surfactants because they are nonionic surfactants and have high HLB values of 16.7 for Tween 20 and 15 for Tween 80. Utilizing nonionic surfactants with high HLB values will expedite the generation of o/w nanoemulsions in the polar medium. The cosurfactant determines the emulsification time in the medium and the size of the nanoemulsion because the cosurfactant molecules will position themselves between the surfactants. Cosurfactants are amphiphilic compounds such as propylene

glycol, polyethylene glycol, and glycol ester, which have an affinity for water and oil phases (Makadia, *et al.*, 2013). Propylene glycol and PEG 400 were chosen as cosurfactants because they can help solubilize hydrophilic surfactants and drugs in an oil base (Amrutkar, *et al.*, 2014).

In this study, surfactant and cosurfactant components with oil were optimized. The results of the optimization were then examined with a UV/Vis spectrophotometer to determine if the preparation had a transmittance value comparable with that of distilled water, and the emulsification duration in media with varying pH was then observed. The data were then evaluated to identify the size, distribution, and zeta potential value of the particles. The purpose of the analysis is to establish whether or not the SNEDDS preparations created meet the criteria for nanoemulsion preparations. It is anticipated that the success of this research will lead to the creation of pharmaceutical formulations containing the active ingredient in the form of bay leaf chloroform extract using the SNEDDS technique as an alternative to effective and efficient oral delivery.

METHODS

Research Material

Bay leaf, ethanol 96%, oleic acid, Tween 80, PEG-400, HCl, NaCl, and aquades.

Research Equipment

Glassware (Pyrex), glass bottle, flakon, magnetic bar, blue tip, 1.5 mL microtube (Stardec), stopwatch, micropipette (Joanlab 100–1000 μ L), oven (Mettler), rotary evaporator (Bibby RE200), analytical balance (Mettler Toledo AG204), moisture analyzer (Ohaus MB23), vortex mixer (VM-300), sonicator (DSA50-GL1-1.81), water bath (Haake DL 30), centrifuge (Hettich Centrifugen Micro 22), magnetic stirrer (IKA® C-MAG HS 7), UV/Vis spectrophotometer (Thermo Scientific), and particle size analyzer (PSA).

Research Methods

Plant Determination

Bay leaf (*Syzygium polyanthum* (Wight) Walp.) authenticity was determined at the Biology Laboratory of Sebelas Maret University in Surakarta. To prepare the chloroform extract of bay leaf (*Syzygium polyanthum* (Wight) Walp.), 500 g of *Simplicia* bay leaf powder was soaked in 4 L of chloroform solvent for 5 days. Then, the macerate was filtered through a glass funnel equipped with a flannel. Then, solvent evaporation was performed using a rotary evaporator with a temperature of 55°C and a rotary speed of 6 until the volume of the macerate was reduced by approximately one-third. Afterward, the macerate that had been evaporated with a rotary evaporator was heated over a water bath at 55°C until it became a thick extract.

Table 1. Ratio of surfactants and cosurfactants.

Composition Ratio	
Surfactant	Cosurfactant
1	1
1	2
1	3
2	3
3	2
2	1
3	1

Optimization of Surfactant and Cosurfactant Composition

Each formula was made as much as 5 mL in flakon. The mixture was homogenized using a magnetic stirrer for 30 min, a sonicator for 15 min, and a water bath at 45°C for 10 min. The results of the mixing were allowed to stand for 24 h at 37°C to see the homogeneity. The formula that remains homogeneous (does not separate) is the formula chosen for the next nanoemulsion formulation.

Optimization of Oil Composition with Surfactants and Cosurfactants

The ratio of surfactants and cosurfactants with a carrier oil (oleic acid) is 4:1. The optimal composition of surfactants and cosurfactants is according to the results of the optimization that has been done previously plus oil (oleic acid). Then, the mixture was homogenized with a magnetic stirrer for 30 min, a sonicator for 15 min, and a water bath at 45°C for 10 min. The results of the mixing were allowed to stand for 24 h at room temperature to see the homogeneity. The formula that remains homogeneous (does not separate) is the formula chosen for the selection of the SNEDDS formula.

Self-Nanoemulsifying Drug Delivery System (SNEDDS) Formula Selection

A total of 100 L of the candidate SNEDDS formula was added with distilled water to a final volume of 50 mL (Patel, *et al.*, 2011a, 2011b),

Table 2. Artificial gastric fluid (AGF) and artificial intestinal fluid (AIF) formulas.

Artificial Gastric Fluid Formula		Artificial Intestinal Fluid Formula	
NaCl	200 mg	MgCl ₂	0.1523 g
HCl 37%	0.7 mL	CaCl ₂	0.1470 g
Aquades	Ad 100 mL	KCl	0.0931 g
		NaCl	1.75850 g
		NaHCO ₃	0.4200 g
		CO ₂ free aquadest	Ad 500 mL
*Condition pH 1.2 *		Condition pH 7	

Table 3. Optimization results of surfactant and cosurfactant composition.

Surfactant	Composition Ratio Surfactant:co-Surfactant	Result	
		PG	PEG
T80	1:1	X	√
	2:1	X	X
	3:1	X	X
	3:2	X	√
	2:3	X	√
	1:3	X	X
	1:2	X	X
T20	1:1	√	X
	2:1	√	X
	3:1	√	X
	3:2	√	X
	2:3	√	X
	1:3	X	X
	1:2	√	X
Composition Ratio Surfactant Combination		T80/T20:PG (1:3)	T80/T20:PEG (1:3)
T80:T20	1:1	X	X
	2:1	X	X
	3:1	X	X
	3:2	X	X
	2:3	X	X
	1:3	X	X
	1:2	X	X

Description: Surfactant=T80 (tween 80), T20 (tween 20); Co-surfactant=PG propylene glycol), PEG (Polyethylene glycol 400); √=homogeneous; X=split (within 24 h).

which was then homogenized using a vortex for 30 s. The result of mixing in the form of a homogeneous emulsion and providing a clear visual appearance is an early sign of the success of making nanoemulsions. Then, using emulsified SNEDDS, its transmittance was measured via spectrophotometry at a wavelength of 650 nm with a blank of distilled water to determine the level of clarity (Patel, *et al.*, 2011a, 2011b). The clearer or closer the transmittance is to the transmittance of distilled water, the larger the emulsion droplets are expected to be in nanometers.

Observation of Emulsification Time

The emulsification time was calculated in three media, namely, distilled water, artificial gastric fluid without pepsin, and artificial intestinal fluid without pancreatin (Table 2). At 37°C, 500 mL of medium was conditioned above a magnetic stirrer rotating at 120 rpm at a speed of 120 rpm. Rapidly, 1 mL of SNEDDS solution was dropped into the medium. Observations were made on the time required from the beginning of the drop until the nanoemulsion was formed. Visual observations were made by looking at nanoemulsion efficiency, transparency, phase separation, and extract droplets.

Table 4. Results of optimization of oil composition with surfactants and cosurfactants.

Surfactant	Cosurfactant	Composition Ratio surfactant: cosurfactant	Result of oil composition: surfactant-cosurfactant (1:4)
T80	PEG	1:1	X
		3:2	X
		2:3	X
T20	PG	1:1	√*1
		2:1	X
		3:1	X
		3:2	X
		2:3	√*2
		1:2	X

Description: Surfactant=T80 (tween 80), T20 (tween 20); Cosurfactant=PG (Propylene glycol), PEG (Polyethylene glycol 400); Oil=Oleic Acid; √=homogeneous; X=split (within 24 h); *(number)=formula code.

The formed nanoemulsion was characterized by the complete dissolution of the SNEDDS herbal extract in the media (Patel, et al., 2011a, 2011b).

Nanoemulsion Droplet Characterization

Using a PSA, the measurements were made to determine the size and distribution of the size and zeta potential of nanoemulsions. To determine the morphology of nanoemulsion particles was visually determined using a transmission electron microscope (TEM).

MTT Assay

Before being exposed to drug treatment, 1×10^4 T47D cells/well were grown in 96-well plates. For cell viability assay, cells were treated for 24 h with an increasing concentration of the SNEDDS of bay leaves. As a negative control, only

a growth medium was added. To each well, 100 µg/mL of MTT solution (0.5 mg/mL in PBS) was added, which was continued with incubation for 3 h at 37°C. The reaction was stopped by dilution with 10% (w/v) sodium dodecyl sulfate in 0.01 N HCl, and cells were incubated overnight. The absorbance was determined by using an ELISA reader at λ 595 nm.

Data Analysis

The results of the optimization of the SNEDDS formula were analyzed visually based on its homogeneity, and the determination of the emulsification time was carried out using a stopwatch, the transmittance value was observed with a UV/Vis spectrophotometer; the particle size and distribution, as well as the zeta potential of the nanoemulsion, were observed using a PSA,

Table 5. Transmittance results of surfactant-cosurfactant and oleic acid composition with a ratio of 4:1.

Formula	Surfactant	Cosurfactant	Surfactant composition ratio: cosurfactant	% transmittance (x±sd)
1	T20	PG	1:1	34.43±0.612*
2			2:3	14.88±0.015

Description: Surfactant=T20 (tween 20); Cosurfactant=PG (Propylene glycol); *(number)=formula code; *=selected formula.

Table 6. The result of transmittance of surfactant-cosurfactant and oleic acid composition with a ratio of 4:1.

Formula	Surfactant	Cosurfactant	Surfactant composition ratio: cosurfactant	% transmittance ($\bar{x} \pm sd$)
I	T20	PG	1:1	83.81 $\pm$ 0.30

Description: Surfactant=T20 (tween 20); Cosurfactant=PG (Propylene glycol).

and the confirmation of nanoemulsion morphology visualization was carried out using a TEM. Then, the result data were compared with the requirements from several existing literature sources to determine the optimal formula and the morphology of the nanoemulsion particles was analyzed descriptively. Maceration using 96% ethanol solvent for 3 days. The macerate was then evaporated using a rotary evaporator at a temperature of 55°C at a speed of 2.5 rpm until a thick extract was obtained. Then, the yield determination, organoleptic test, and moisture content test were conducted.

Table 7. Observation of emulsification time at 37°C.

Formula	Media	Emulsification time (second) ($\bar{x} \pm sd$)
I	Akuades	34.17 $\pm$ 0.8802
	AGF	103.25 $\pm$ 0.9725
	AIF	100.17 $\pm$ 0.0814

Description: AGF=artificial gastric fluid; AIF=artificial intestinal fluid.

Graphs of cell viability versus sample concentration are created using linear regression equations, with the resulting equation function $y = bx + a$ used to calculate the IC_{50} value. IC_{50} value was analyzed statistically using Microsoft Excel, and statistical significance was estimated by using the analysis of variance test. Statistical significance was set at $p < 0.05$.

RESULTS

Extraction with 500 g of Simplicia bay leaf powder macerated using 4 L of chloroform solvent resulted in a thick extract of 28.37 g with an extract yield of 5.647%.

The results of optimization of the composition of surfactants and cosurfactants (Table 3) show that to produce a homogeneous mixture a ratio of 1:1; 3:2 and 2:3 between Tween 80 and PEG 400. Ratio 1:3 of Tween 20 and propylene glycol could not produce a homogeneous mixture.

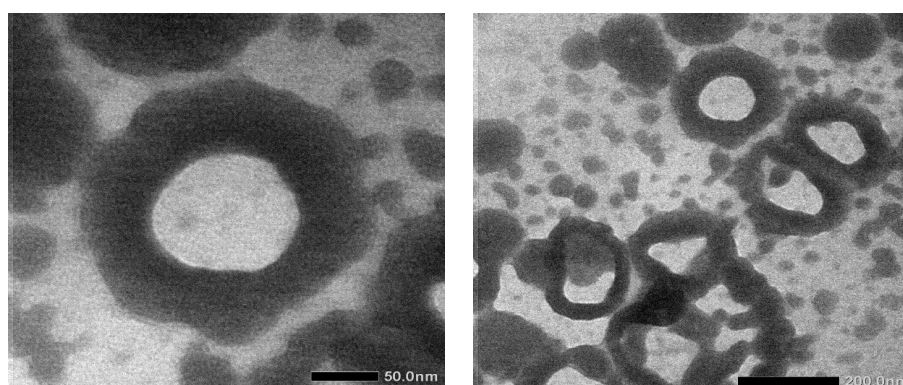


Figure 1. Results of transmission electron microscope (TEM).

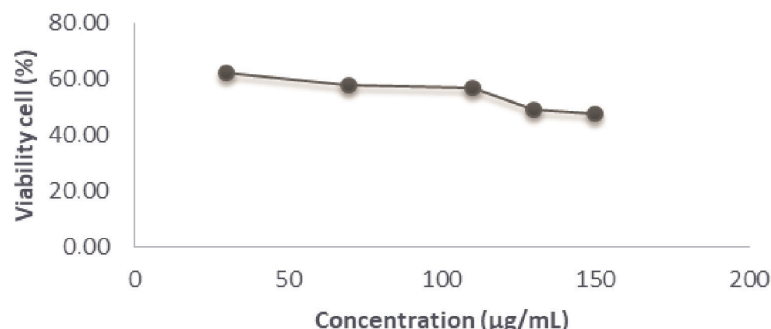


Figure 1. Linear regression equation between extract concentration versus % cell viability treatment of SNEDDS with a concentration series of 30; 70; 110; 130; 150 µg/mL with respect to T47D cell.

Table 5 shows the results of the observation of transmittance (%) at a wavelength of 650 nm. Based on the results in Table 6, formula 1 yields a three-time average transmittance value of 83.81%.

Observation of Emulsification Time

Based on the results in Table 7, SNEDDS formula 1 was able to form nanoemulsions in distilled water media with an average of 34.17 s, whereas in AGF media it took an average of 103.25 s and 100.17 s in AIF media.

Nanoemulsion Droplet Characterization

Based on the results in Table 8, the nanoemulsion droplet size was in the range of 50–500

nm with the polydispersity index (PI) value of the nanoemulsion droplets less than 1, and the nanoemulsion droplets were distributed in the range of 35.03–454.69 nm. Based on the results in Table 9 show that SNEDDS formula 1 has a zeta potential value not greater than +30 mV and not less than –30 mV.

Nanoemulsion Morphology Visualization Test

Figure 1 shows that the shape of the nanoemulsion particles produced is spherical, although there are still particles that are less spherical so that the contact between the particles does not form aggregates.

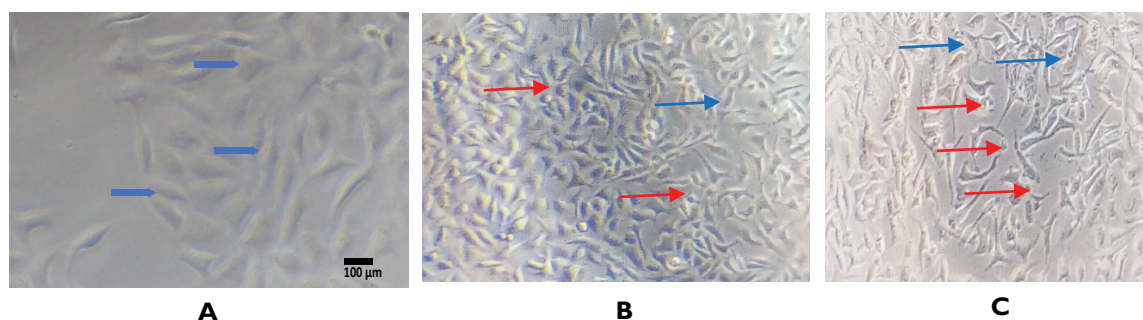


Figure 2. Effect of treatment of SNEDDS bay leaves on T47D cells (1×10^4 cell/well) after 24 h with 100x magnification, using DMEM High glucose media in 96well-plate shows decreased viability of T47D cells which is directly proportional to increased SNEDDS concentration. (A) cell control; (B) SNEDDS bay leaves concentration of 130 µg/mL; (C) SNEDDS bay leaves concentration of 150 µg/mL. Blue arrows indicate living cells, red arrows indicate dead cells.

Table 8. Nanoemulsion droplet size and polydispersity index value.

Formula	Droplet size (nm)	Polydispersity index (PI) droplet
I	165.5	0.198

Formula I: 2 mL of Tween 20, 2 mL of Propylene Glycol, 1 mL of Oleic Acid and 100 mg of Bay leaf extract.

Effect of Self-Nanoemulsifying Drug Delivery System (SNEDDS) from Chloroform Extract of Bay Leaf (*Syzygium Polyanthum* (Wight) Walp.) with Oleic Acid as a Carrier Against T47D Cell Growth

The cytotoxic effect test on T47D cells is a preliminary test using a culture of T47D cells in 96-well plates with the MTT assay method whose absorption results will give a purple color and read the absorbance using the ELISA reader. The absorbance results are used to calculate the IC_{50} (inhibition concentration) value, which is the ability of a compound that can cause growth inhibition in 50% of the cell population. IC_{50} values obtained in the treatment of SNEDDS were 138 μ g/mL. Figure 1 presents the % viability results of extracted cells.

Based on the linear regression graph, the function equation was obtained: $y = -0.1193x + 66.47$ with an R^2 value amounting to 0.90. The IC_{50} value was obtained at 138 μ g/mL. The cytotoxic effects of the treatment of SNEDDS from bay leaves are categorized as cytotoxic moderate so that it can be used as a chemopreventive agent. Figure 3 shows the morphological profile of T47D cell growth in the treatment of SNEDDS from bay leaves.

Table 9. Nanoemulsion droplet zeta potential.

Formula	Nanoemulsion droplet zeta potential (mV)
I	-0.4

DISCUSSION

Various ratios of surfactants, propylene glycol, and PEG 400 cannot be used to generate a homogenous mixture. According to the results of optimization of oil composition with surfactant–cosurfactant (Table 4), a 1:1 and 2:3 mixture of Tween 20 and propylene glycol can produce a homogenous mixture. Based on these results, oleic acid as a carrier oil mixed better with Tween 20 than with Tween 80. The selection of the SNEDDS formula was performed on the formulas chosen in the preceding optimization phase, namely, formulas 1 and 2.

Table 5 shows that both formulas have a three-time average transmittance value, which is far from the transmittance value of distilled water (more than 75%). This is because the ratio of surfactant–cosurfactant is too small compared with oil; hence, the ratio of the composition ratio of surfactant–cosurfactant and oil is increased to 9:1.

The transmittance value shows a value of more than 75%, which indicates the droplet size formed by oil in water, is getting smaller, so it is estimated to have a droplet size of approximately 50–500 nm. Then, from the results of the transmittance test, the formula that has the transmittance value closest to the transmittance of distilled water (more than 75%) is chosen to observe the emulsification time.

Observation of Emulsification Time

According to research conducted by Meirista (2014), the emulsification time requirement for SNEDDS preparations is less than 5 min. This shows that formula 1 can give an emulsification time of less than 5 min in the three media.

Nanoemulsion Droplet Characterization

The requirement for droplet size for SNEDDS nanoemulsion preparations is between 50 and 500 nm (Shakeel, *et al.*, 2008). Table 8 shows

that the PI value of the nanoemulsion droplets is less than 1 and the nanoemulsion droplets are distributed in the range of 35.03–454.69 nm. A PI value of less than 1 serves as an indicator of homogeneous size distribution.

The low zeta potential causes the attraction between the dispersion particles to exceed the repulsion; hence, the possibility of flocculation is greater. However, because the preparation is in the form of SNEDDS, it is judged suitable because it is more stable than nanoemulsion; the preparation in the form of SNEDDS will only become emulsified upon consumption and interaction with gastric fluid.

Nanoemulsion Morphology Visualization Test

If the shape of the nanoemulsion particles is less spherical, it will facilitate the contact between the particles, leading to aggregation (Couvreur, et al., 2002). Based on the research, it was found that to obtain the SNEDDS formula of bay leaf chloroform extract with oleic acid as a carrier oil that met the criteria for nanoemulsion preparation, it was necessary to have a much smaller ratio of oil composition (oleic acid) than surfactants and cosurfactants.

The greater the IC_{50} value of a compound, the more toxic it is because it requires a large concentration to inhibit cell growth; conversely, the smaller the IC_{50} value, the greater the potential for oxidation to cells because the required concentration is small. Cytotoxicity tests can provide information regarding the concentration of a compound that still allows cells to survive. The results of a single cytotoxic test of SNEDDS bay leaves showed moderate cytotoxic effects on the T47D cell line. A compound that has an IC_{50} value of $<100 \mu\text{g/mL}$ is a compound with a potential cytotoxic effect, and a compound that has an IC_{50} value of $100\text{--}1000 \mu\text{g/mL}$ can be said to be a compound with a moderate cytotoxic effect. The compound has no cytotoxic effect if the IC_{50} value is $>1000 \mu\text{g/mL}$.

CONCLUSION

(1) Optimization of surfactant and cosurfactant composition from SNEDDS chloroform extract of Salam leaf (*Syzygium polyanthum* (Wight) Walp.) can produce a homogeneous phase. (2) Optimization of surfactant and cosurfactant composition with oil (oleic acid) from SNEDDS chloroform extract of Salam leaf (*Syzygium polyanthum* (Wight) Walp.) can produce a homogeneous phase. (3) Optimization of surfactant and cosurfactant composition with oleic acid from SNEDDS chloroform extract of Salam leaf (*Syzygium polyanthum* (Wight) Walp.) resulted in an optimal formula with a composition ratio of Tween 20:propylene glycol:oleic acid=2.25:2.25:0.5, which meet several criteria for nanoemulsion preparations including emulsification time of less than 5 min and having a particle size of 165.5 nm with a PI value of less than 1 although the preparation is less stable as indicated by a zeta potential value not greater than +30 mV and not less than -30 mV and has a spherical particle shape.

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