

## Formulation and Antioxidant Property of Bitter Melon Seed Oil Loaded into SNEDDS as A Nutraceutical

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### ABSTRACT

Bitter melon seed oil has an antioxidant activity which is beneficial to treat various degenerative diseases. However, the bitter melon seed oil is less soluble in the gastrointestinal tract and has low absorption. Therefore, a self-nanoemulsion dosage form is needed to support its absorption and maintain its antioxidant activity. This study aimed to formulate bitter melon seed oil into a self-nano emulsifying drug delivery system (SNEDDS) and examine its antioxidant activity change after being transformed into SNEDDS using the Ferric Reducing Antioxidant Power (FRAP) method and DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The SNEDDS formulation uses bitter melon seed oil as the active ingredient and the oil phase, Cremophor RH 40 as a surfactant, and glycerin as a co-surfactant. The results showed that the best SNEDDS formula has a ratio of oil: Smix (surfactants mixture) of 1:4. The best formula transmittance was  $97.35 \pm 0.04\%$  with an emulsification time of  $15.69 \pm 0.06$  seconds, a pH value of  $6.87 \pm 0.08$ , and a particle size of  $31.8 \pm 16.3$  nm. Thermodynamic stability and robustness to dilution tests showed that the preparation was stable and resistant to various dilutions and pH. The antioxidant activity of bitter melon seed oil before and after being transformed into SNEDDS resulted in a statistically insignificant difference. This study concluded that bitter melon seed oil SNEDDS has good physical characteristics, stability, and no antioxidant activity changes after formulation, supporting its application to new therapeutic uses.

**Keywords:** Bitter melon seed oil, nutraceutical, SNEDDS, antioxidant activity.

### INTRODUCTION

Nutraceutical is food or part of food that is useful as a nutrient and medicinal to prevent and cure diseases, for example, Parkinson's, osteoarthritis, allergies, Alzheimer's, Cardiovascular Disease, cancer, inflammation, obesity, and diabetes mellitus (Chauhan *et al.*, 2013; Dutta *et al.*, 2018). Bitter melon seed oil is one of the nutritional agents containing PUFAs (Poly Unsaturated Fatty Acids), such as conjugated  $\alpha$ -linoleic acid, which acts as a source of serum lipid activity *in vitro* and *in vivo*, reduces body weight, and lowers blood glucose levels (Anjum, 2012; de Moraes *et al.*, 2017). Other bioactive compounds are minerals (F, K, Mg, S, Ca), flavonoids, tocopherols, and phenolics (Anjum, 2012). Phenol is an antioxidant that can scavenge free radicals and reduce diabetes complications by reducing ROS (reactive oxygen species), TNF- $\alpha$ , and oxidative stress (Widowati, 2008). This phenolic compound is the basis for researchers to study antioxidant activity.

The bitter melon seed oil has low water solubility, hence is difficult to dissolve in the digestive tract (Parmar *et al.*, 2015), is less permeable, and has low bioavailability. The bioavailability of organic compounds can be improved using colloid nanoemulsion systems. A waterless preconcentrate nanoemulsion system, SNEDDS, was used to overcome this weakness. SNEDDS is an oil or fat-based nanoemulsion formula consisting of a mixture of drugs, oils, surfactants, and co-surfactants to produce very fine oil droplets with light stirring (Parmar *et al.*, 2015).

Previous studies showed that SNEDDS was able to improve drug release and increase the activity of active ingredients. The previous study showed that pomegranate seed oil SNEDDS are suitable for functional food and clinical fields. SNEDDS can increase the inhibition of pomegranate seed oil to breast cancer MCF-7 up to 2.03 folds *in vitro* (Lu *et al.*, 2015). The combination

of piperine and curcumin formulated in SNEDDS preparations then solidified using Neusilin can also increase dissolution up to 77% (Kazi *et al.*, 2020).

Based on the description above, it is necessary to research the bitter melon seed oil formulation into SNEDDS to maintain its antioxidant and improve its solubility. There is no study that reported bitter melon seed oil SNEDDS before. Therefore, developing the SNEDDS formula for bitter melon seed oil is necessary to test its antioxidant activity using the FRAP method and DPPH assay.

## MATERIALS AND METHODS

The bitter melon seed oil phase was purchased from Nature in Bottle (India), tween 20, tween 80, Cremophor RH 40, PEG 400, propylene glycol, glycerin, and other chemicals were pharmaceutical grade.

### Selection of oil phase

In this study, bitter melon seed oil was used as an active ingredient and an oil component in SNEDDS.

### Selection of surfactants

Co-surfactant was selected from three candidates: Tween 20, Tween 80, and Cremophor RH 40. Each of the surfactant candidates was weighed 300mg and added to 300mg of bitter melon oil. Fifty milligrams of the mixture were dissolved in 50mL of distilled water. The ease of emulsification and transmittance percentage is the basis for selecting the surfactants (Patel *et al.*, 2011).

### Selection of co-surfactants

Co-surfactants used in this study were propylene glycol, PEG 400, and glycerin. Each co-surfactant was weighed 100mg, then 200mg of selected surfactants were added, and the mixture was added with 300 mg of bitter melon seed oil. A total of 50mg of the mixture was dissolved in 50mL distilled water and left for 2h. The co-surfactant candidates were selected based on the percentage of transmittance and the amount of inversion to form nanoemulsions (Patel *et al.*, 2011).

### Selection of surfactant and co-surfactant

The surfactant and co-surfactant mixture (1:1; 1:2; 1:3; 2:3; 3:2; 2:1; 3:1) was homogenized using a magnetic stirrer for five minutes and left at room temperature 24h. The mixture was then visually observed. Mixtures that remained stable

(not separating) were selected for the SNEDDS formulation.

### Selection of oil, surfactant, and co-surfactant mixture

Mixtures of oil: Smix in the ratio of 1:10, 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3, 1:2, 1:1 were emulsified with distilled water. The resulted nanoemulsion was evaluated based on criteria following Poorani *et al.* (2016). Grade A mixture was chosen for further testing.

### Characterization of bitter melon seed oil SNEDDS

#### *Organoleptic*

The bitter melon seed oil SNEDDS was yellow, transparent, and had an oil aroma.

#### *Percentage of transmittance*

Fifty milligrams of bitter melon seed oil SNEDDS dissolved in 50 mL, then the percentage of transmittance was measured using a UV-Vis spectrophotometer with a wavelength of 650nm (Kane *et al.*, 2016).

#### *Emulsification time*

This study was performed visually by pipetting 50 $\mu$ L SNEDDS into 50 mL distilled water stirred at 37°C, 100 rpm. The time taken to form nanoemulsions spontaneously was recorded (Savale, 2015).

#### *pH measurement*

One milliliter SNEDDS preparation was added with distilled water then the nanoemulsion formed was tested using a pH meter.

#### *Particle size determination*

This particle size measurement used a Particle Size Analyzer (PSA) by adding SNEDDS with distilled water (1:1000). The expected SNEDDS particle size is <200nm (Wahyuningsih *et al.*, 2015).

#### *Thermodynamic stability*

#### *Centrifugation test*

The SNEDDS formula was centrifuged at a speed of 5000 rpm within 30 minutes. Precipitation and visual phase separation were observed (Savale, 2015).

#### *Heating-cooling cycle*

The SNEDDS formula was stored at 4°C and 45°C for at least 24h and visually observed for precipitation and phase separation. This study was repeated in three cycles (Savale, 2015).

#### *Freeze-thaw cycle*

The SNEDDS formula was stored at -20°C and 25°C for at least 24h and visually observed for

precipitation and phase separation. This test was repeated in 3 cycles (Savale, 2015).

**Robustness to dilution**

The study is carried out by diluting 50x and 1000x on SNEDDS formula using 0.1N HCl (pH 1.2, simulated gastric fluid), phosphate buffer (pH 6.8, simulated intestinal fluid), and distilled water (Reddy, 2018). The percentage of transmittance was read with a UV-Vis spectrophotometer at the wavelength of 650nm.

**Antioxidant activity study FRAP method**

The method of antioxidant oil analysis was adopted from Emerenciano *et al.*, 2019 and Rafi *et al.*, 2020. The percentage of reducing power calculate using equation 1.

$$\text{Reducing power} = \left( \frac{A1-A0}{A2-A0} \right) \times 100\% \dots\dots\dots 1$$

Description: A0 = absorbance of the blank solution ; A1 = absorbance of the test sample; 2 = Absorbance of ascorbic acid

**DPPH assay**

The DPPH assay test was carried out according to the method used by Lu *et al.* (2020). The antioxidant activity of bitter melon seed oil was determined by diluting bitter melon seed oil to 800µL with ethanol and mixed with 200µL of isopropanol and 2.0mL of DPPH. The final oil concentration ranges from 0.08 to 0.24%v/v. The solution was left in the dark for 30min, then the absorbance at the maximum wavelength was determined (A). As the control, 1.0mL of ethanol was mixed with 2.0mL of DPPH, and the absorbance measurement was performed as mentioned above (A0). Calculation of the antioxidant activity was done using equation (2).

$$\text{Scavenging rate} = \frac{(A0-A)}{A0} \times 100\% \dots\dots\dots 2$$

Determination of the antioxidant activity of bitter melon seed oil SNEDDS (0.1-1.0% (v/v)) was done by diluting SNEDDS with water, then 1.0mL was mixed with 2.0mL DPPH in ethanol, and the absorbance was measured at 517 nm. In addition, the absorption of 1.0mL of SNEDDS and 2.0mL of solvent (Ab) was also determined. One mL of SNEDDS blank was mixed with 2.0mL of DPPH, and the absorption was determined (A0). The scavenging rate was calculated using equation 3.

$$\text{Scavenging rate} = \frac{(A0-(A-Ab))}{A0} \times 100\% \dots\dots\dots 3$$

IC<sub>50</sub> calculated from the graph was obtained from the linear regression of the percentage of inhibition against the various concentrations of the oil. It shows a 50% reduction in DPPH activity.

**Statistical analysis**

Statistical analysis was performed using the independent t-test and One-Way ANOVA. The statistical analysis used a confidence level of 95%. If a p-value <0.05, then it is significantly different, whereas a p-value >0.05 is not significantly different.

**RESULTS AND DISCUSSION**

**Oil phase**

Other oils than bitter melon seed oil were not used because they could reduce the loading capacity of oil in the SNEDDS system. According to Nasr *et al.* (2016b), the oil phase in a high concentration could produce a large droplet size and lower transmittance percentage (Ruan *et al.*, 2010). Therefore, the highest amount of oil to be used was selected by considering the clarity of the nanoemulsion produced.

**Surfactant selection**

Cremophor RH 40 produced the highest % transmittance and eased the nanoemulsion production compared with Tween 20 and Tween 80. Cremophor RH 40 is a surfactant with a hydrophilic-lipophilic balance (HLB) of 14-16, which contains about 75% hydrophobic and 25% hydrophilic parts. The hydrophobic portion consists mainly of the fatty acid ester of glycerol, polyethylene glycol, and the fatty acid ester of polyethylene glycol. In contrast, the hydrophilic portion contains polyethylene glycol and glycerol ethoxylate (Rowe *et al.*, 2009).

From its polymer chain composition (HLB), Cremophor RH 40 has a high loading capacity to emulsify oil in water. For this reason, Cremophor RH 40 is the most oil-soluble to form nanoemulsion compared to Tween 20 and Tween 80. Therefore, the surfactant candidate chosen for the SNEDDS formulation of the bitter melon seed oil was Cremophor RH 40.

**Co-surfactant selection**

Glycerin is an amphiphilic solvent often used in SNEDDS preparation. It could increase drug loading and shorten the time needed to form nanoemulsions (Date *et al.*, 2010; Patel *et al.*, 2011; Winarti *et al.*, 2018). Glycerin produced the highest percentage of transmittance and ease the formation of nanoemulsion among other co-surfactant candidates. Therefore, glycerin was chosen as a co-surfactant.

Table I. The mixture of bitter melon seed oil and Smix

SNEDDS	Parameters		Grade
	Emulsification time (second)	Visual appearance	
(1:10)	12	Transparent	A
(1:9)	13	Transparent	A
(1:8)	16	Transparent	A
(1:7)	16	Transparent	A
(1:6)	29	Transparent	A
(1:5)	30	Transparent	A
(1:4)	36	Transparent	A
(1:3)	60	White	B
(1:2)	60	White	B
(1:1)	120	White	B

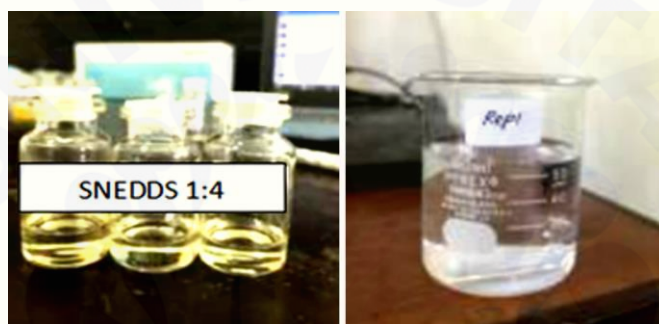


Figure 1. Bitter melon seed oil SNEDDS (A) Before dilution; (B) After dilution.

Table II. Physical characterization of bitter melon seed oil SNEDDS

Physical characterizations	Results
Organoleptic	yellow, clear, and distinctive aroma of bitter melon oil
% transmittance	97.35±0.04%
Emulsifying time	15.69±0.06
pH	6.87±0.08
Particle size	31.8±16.3
Polydispersity index	0.035
Thermodynamic stability	Stable
Robustness to dilution	Stable

### Selection of surfactant and co-surfactant mixture (Smix)

This test aimed to study the Smix, which remained stable with varying ratios. After leaving for 24h, the 1:1 mixture did not separate. It remained clear compared to other ratios. Because surfactant was added with the co-surfactant at the same amount, the nanoemulsion formed was much better than the other ratios. This 1:1 mixture could reduce the interface stress and increase interface fluidity (Nasr *et al.*, 2016a).

### Oil and selected Smix mixture

According to the grading system, the mixture of bitter melon seed oils with Smix in various ratios was recorded (Table I). The oil: Smix ratio selected in this study was 1: 4 because it had the highest oil content and the lowest Smix than other formulas in grade A (Figure 1).

The high oil content causes the SNEDDS formula to have a high loading capacity of bitter melon seed oil. At the same time, Smix is chosen as the lowest because it contains surfactants which in

large amounts can be irritating and toxic (Rowe *et al.*, 2009). This selected ratio is capable of forming a clear nanoemulsion.

### Organoleptic

The SNEDDS formula of bitter melon seed oil produced a yellow, clear, and distinctive aroma of bitter melon oil (Table II).

### The percentage of transmittance

Transmittance can indicate the formation of nanoemulsions (Table II). The bitter melon seed oil SNEDDS showed a high % transmittance ( $97.35 \pm 0.04\%$ ), indicates the formula's clarity and droplet size close to the nanometer range (Bali *et al.*, 2011).

### Emulsification time

Emulsification time determines the ease of nanoemulsion formation in light agitation (Patel *et al.*, 2011). The bitter melon seed oil SNEDDS with a 1:4 ratio could form nanoemulsions with an average time of 15.65-15.73 seconds (Table II).

The emulsification time was mediated by the performance and proportion of Cremophor RH 40 and glycerin. In this case, Cremophor RH 40 and glycerin played a role in forming the water and oil interface layer. Therefore, it affected the time required for emulsion formation.

### pH measurement

According to Zhao (2015), the SNEDDS pH value in the range of 6.5-9.0 can significantly influence colloid systems' stability by affecting the ionization and surface charge of the droplets (Xue *et al.*, 2018). The SNEDDS formulation of bitter melon seed oil exhibited a slightly acidic pH ( $6.87 \pm 0.08$ ), making it suitable for biological applications (Table II).

The bitter melon seed oil used had a significant fatty acid composition of alpha-eleostearic acid (53.30%), palmitic acid (2.6%), stearic acid (28.50%), oleic acid (5.4%), and linoleic acid (9.10%). The bitter melon seed oil SNEDDS was slightly acidic, probably because the SNEDDS formula contained the bitter melon seed oil in a small amount (20%).

### Determination of particle size

Nanoemulsion size determines the speed and amount of drug dissolved and absorbed in the digestive tract (Zhao, 2015). The small particle size has a large surface area to increase drug absorption (Syukri *et al.*, 2018). According to Wang *et*

*al.* (2009), an acceptable criterion for nanoemulsion size is around 20-200nm. The SNEDDS particle size had an average droplet size of  $31.8 \pm 16.3$  nm (Table II), so they were in the specified range (Figure 2).

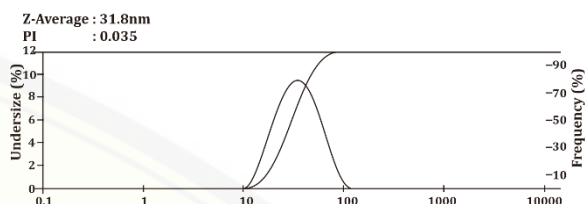


Figure 2. Particle size and size distribution of bitter melon seed oil SNEDDS

Droplet size and droplet size distribution are the main factors affecting product properties, such as rheology, appearance, chemical reactivity, stability, and physical properties (McClements, 2012; Tardos *et al.*, 2004). The Ostwald Ripening is an instability phenomenon involving large droplets that grow by combining tiny droplets. Monodisperse droplets of nanoemulsion will be less affected by Ostwald Ripening. The SNEDDS formula of bitter melon seed oil has a polydispersity index of 0.035, which shows a uniform size. Therefore, Ostwald Ripening does not affect the nanoemulsion stability.

### Thermodynamic stability

A stability test determines SNEDDS preparation's stability. A cycling test could determine the crystal forming. Surfactants in a solution can form liquid crystalline phases caused by geometric aggregation due to external influences such as temperature, pressure, and flow (Manero *et al.*, 2010). However, crystals were not found in the SNEDDS formula due to Cremophor RH 40 as an emulsifier, which can maintain emulsion stability. Cremophor RH 40 inhibits coalescence, creaming, and phase separation processes. Therefore, the bitter melon seed oil SNEDDS is a stable preparation (Table II).

### Robustness to dilution

This test determines the resistance of nanoemulsion in various volumes and pH (1.2; 6.8; distilled water). The resistance of the nanoemulsion system is seen from the results of the % transmittance of the samples that were not significantly different ( $p\text{-value} > 0.05$ ) in the One-Way ANOVA test and the Independent Sample t-test (Table II). This study shows that the body's

drug delivery system is also stable (Nasr *et al.*, 2016a).

**Antioxidant activity study**

*FRAP Method*

The antioxidant activity in bitter melon seed oil decreased after formulation (Table III), possibly due to storage factors that cause autooxidation such as oxygen, light, temperature, and the container used to store preparations (Juniarka *et al.*, 2011). Nanoemulsions have a large specific surface area and allow chemical degradation at the oil-water interface, such as lipid oxidation. This autooxidation can be accelerated because no antioxidants were added to the preparation.

Table III. Antioxidant Activity

Sample	Absorbance		
	Ascorbic Acid	Oil	SNEDDS
Replication 1	0.24	0.20	0.18
Replication 2	0.25	0.18	0.17
Replication 3	0.23	0.17	0.15
Mean ± SD	0.24± 0.01	0.18± 0.02	0.16± 0.02
% Reducing Power	100± 8.78%	64.80 ± 13.41%	54.45± 13.42%

Percentage of vitamin C's reducing power as standard (40µg/mL), bitter melon seed oil, and SNEDDS were analyzed using One-Way ANOVA. The results showed a difference in the three variables' average, with a p-value of <0.05. The post hoc test showed that ascorbic acid and bitter melon seed oil were significantly different in reducing power. In contrast, the bitter melon seed oil before and after being formulated into SNEDDS did not differ significantly.

*DPPH Assay*

In contrast with the FRAP method, the DPPH assay showed an increase in the antioxidant activity of bitter melon seed oil after being formulated into SNEDDS preparations. However, the results of the t-test showed no significant difference.

The IC-50 of bitter melon seed oil and bitter melon seed oil SNEDDS can be seen in Table IV. The previous study showed that the IC-50 of bitter melon seed oil was 11.31 ± 0.77 mg/ml, and the phenol content of bitter melon seed oil was 0.0118 ±0.0006%. The flavonoid content was 0.0127±0.0004%. Qualitative phytochemical screening showed bitter melon seed oil contains flavonoids, triterpenoids, and alkaloids (Winarti *et*

*al.*, 2021). The IC50 results in this study were different from the previous study because of the different methods used. The method before using a methanol extraction, but in this study, the oil was dissolved in ethanol and isopropanol. From this dissolved oil, more antioxidant compounds can be measured.

Table IV. Antioxidant Activity

The samples	IC <sub>50</sub> (mg/mL)
Bitter melon seed oil	2.28±0.011
Bitter melon seed oil SNEDDS	2.10±0.020

SNEDDS could increase the antioxidant activity because glycerides and phospholipids (Seow *et al.*, 2014) in the oil have some characteristics of surfactants, hence strengthening the interaction among the oil, surfactant, and co-surfactant of SNEDDS. This strong interaction leads to bitter melon seed oil contact DPPH and improves radical scavenging (Lu *et al.*, 2020).

Khoirunnisa and Miladiyah's (2019) finding showed an increase in the antioxidant activity of the SNEDDS formulation of black cumin seeds extract evaluated by the DPPH. The % inhibition of non-SNEDDS black cumin seeds extract was about 2.6% and after being formulated into SNEDDS preparation increased to 73%. Wahyuningsih and Khoirunnisa (2019) showed no influence of SNEDDS formulation on the histopathology of DMBA-induced lung organs compared with Black cumin seed oil administration only. These different results might be influenced by the physical properties of the nanoemulsion, the stability of the molecule, and the partition of the antioxidant compounds in the lipid phase (Jusnita and Syurya, 2019).

**CONCLUSION**

The SNEDDS formula with the bitter melon seed oil and surfactant mixture (1: 4) was the best formula chosen because it met the nanoemulsion criteria and was stable. *In vitro* antioxidant test for the bitter melon seed oil SNEDDS was proven no different from bitter melon seed oil only. These results suggest that SNEDDS is suitable for the bitter melon seed oil formulation to be widely applied to new therapeutic uses.

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