Toxicity of Self-nanoemulsifying Drug Delivery System Formulation of *Nigella Sativa* L. Seed Oil against Adult *Danio rerio*

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Abstract: *Nigella sativa* L. (*N. sativa* L.) has been used in traditional medicine due to its numerous therapeutic effects, but its oral form has low solubility and has a minor therapeutic effect. The development of self-nanoemulsifying drug delivery system (SNEDDS) formulation for *N. sativa* L may solve this issue. The pharmacological activity of the SNEDDS formulation of *N. sativa* L. seed oil extract (NSOE) has been widely explored but its toxicity remains unknown. This study aimed to determine the lethal concentration of NSOE SNEDDS formulation. An experimental study was conducted using adult zebrafish, aged 4–6 months, incubated with NSOE SNEDDS formulation at 500, 250, 125, 62.5, and 31.5 part per million (ppm) concentrations and with non-SNEDDS formulation at 125, 62.5, 31.25, 15.625, and 7.8125 ppm concentrations. The mortality was calculated through macroscopic examination after 24, 48, 72, and 96 hours of exposure. The half-maximal lethal concentration (LC_{50}) of the NSOE SNEDDS and non-SNEDDS formulations was determined using the probit analysis. The LC_{50} of NSOE SNEDDS formulations was 154.637 $\pm$ 75.609 ppm and was not significantly different from that of non-SNEDDS (72.358 $\pm$ 15.253 ppm) ($p = 0.138$). The toxicity of the SNEDDS formulation of NSOE was comparable to that of the non-SNEDDS.

1 INTRODUCTION

Nigella sativa L. (*N. sativa* L.) is a herbal plant from the Ranunculaceae family and is widely grown in Mediterranean countries, the Middle East, Eastern Europe, and West Asia (Abedi et al., 2017). Several phytochemical studies of *N. sativa* L. showed that its extract contains numerous antioxidant compounds, including thymoquinone, carvacrol, t-anethole, and 4-terpineol, which indirectly reduce reactive oxygen species production and inhibit lipid peroxidation (Amina, 2016). Thymoquinone has the most powerful antioxidant properties (Kooti et al., 2016).

Herbal medicines are usually orally administered since this route of administration is the safest, most convenient, and most inexpensive (Cherniakov et al., 2015). However, the low solubility and poor oral bioavailability of many herbal medicines lead to less optimal effectiveness. Therefore, researchers have begun developing oil-based drug formulations in the form of nanoemulsions that are expected to improve the oral bioavailability and drug solubility of herbal plant extracts, including the development of a self-nanoemulsifying drug delivery system (SNEDDS) (Abdelbary et al., 2013).

The SNEDDS formulation consists of a mixture of isotropic oils, surfactants, and cosurfactants that are capable of spontaneously forming oil nanoemulsions in the gastrointestinal tract by producing nanometer-sized droplets (<300 nm in size) when dispersed in liquid media (Christophersen et al., 2014; Patel et al., 2011) Drug preparations in SNEDDS formulations have several advantages, including the ability to maximize absorption and transportation, modulate drug biodistribution and disposition, and allow targeted drug delivery to reduce the side effects. SNEDDS formulations tend to be more physically and chemically stable for long-term storage and allow the packaging of drugs in unit dosage forms, using hydroxypropyl methylcellulose or both soft and hard gelatin capsules, than other nanoemulsion systems (Date et al., 2010).

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Previous studies have focused on the pharmacological effectiveness of SNEDDS formulations; however, their toxicity in living cells, including the SNEDDS formulation of *N. sativa* L. seed oil extract (NSOE), has not been widely investigated. Toxic impacts are important and inseparable from the development of new drugs (Parasuraman, 2011). Thus, this study aimed to determine the toxicity of NSOE in a SNEDDS formulation in comparison with the toxicity of the non-SNEDDS formulation on adult zebrafish.

2 MATERIALS AND METHODS

This study was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine of Universitas Islam Indonesia with protocol Number 37/Ka.Kom.Et/70/KE/V/2019.

2.1 Animal Subjects

Both male and female adult zebrafish (*Danio rerio*) were used in the study. The fish were 4–6 months old and healthy, as characterized by active swimming. The fishes that died before the research took place were excluded. Prior to treatment, the fish were adapted for one week. Zebrafish were identified at the Biology Research Center, Indonesia Academy of Science at Bogor, Indonesia.

2.2 Preparation of *N. sativa* L. Seed Oil Extract and SNEDDS Formulation

NSOE was prepared following the previous study by Akrom et al. (Akrom & Fatimah, 2015). *N. sativa* L. seeds were dried and mashed to form a powder. Overall, 1 kg *N. sativa* L. seeds was soaked in 1 L of 70% ethanol for 48 hours and filtered to separate the macerated yield from the residue. The product of the maceration process was collected, and an evaporator was used to remove the 70% ethanol solvent and form a thick NSOE. The viscous NSOE was kept upright until it divided into two phases, the extract and the oil phases. The oil phase was then used for the SNEDDS formulation.

In this study, NSOE was produced with the maceration method using 70% ethyl alcohol. This method is simple, easy to perform, inexpensive, and can protect the thermolabile compounds in *N. sativa* L. seed from damage (Savitri et al., 2017). Different concentrations of surfactants and cosurfactants were added to the oil phase after maceration to prepare the SNEDDS formulation. The determination of the optimal NSOE SNEDDS was based on previous research (Wahyuningsih & Putranti, 2015), in which the best composition was NSOE 0.154 parts, Tween 80 0.587 parts, and polyethylene glycol 400 (PEG 400) 0.259 parts. Therefore, the formulation prepared consisted of 0.532 mL of NSOE, 2.047 mL of Tween 80 (surfactant), and 0.258 mL of PEG 400 (cosurfactant) (Wahyuningsih & Putranti, 2015)

2.3 Formulation Stability Test

Stability of SNEDDS formuations was evaluated using heating-stability, freeze-thaw, and centrifugation tests (Senapati et al., 2016). The heating-stability test was conducted by storing SNEDDS samples in a refrigerator at 4°C for 24 hours and then by transferring them to an incubator at 40°C for 24 hours (48 hours per cycle). The freeze-thaw test was conducted by storing SNEDDS samples at temperatures between −21°C and 25°C for 48 hours. The centrifugation test was performed by centrifuging SNEDDS samples at 5000 rpm for 30 minutes. All tests were repeated six times (six cycles); then, organoleptic observations and instability parameters (phase separation and precipitation) were recorded.

2.4 Determination of Globule Size, Zeta Potential, Polydispersity Index, and %Transmittance

The NSOE SNEDDS formulation was diluted with water at a ratio of 1:25 on a magnetic stirrer until a nanoemulsion was formed. The nanoemulsion was then put into a cuvette and measured using a Particle Size Analyzer (SZ 100, HORIBA) to determine globule size, zeta potential, and polydispersity index (PDI). The %transmittance was measured by adding 5 mL distilled water to 0.1 mL SNEDDS formulation of NSOE and then rotating them in a vortex for 30 seconds. The transmittance was read using a UV-Vis spectrophotometer (UV-Vis UH5300, Hitachi) at a wavelength of 650 nm with distilled water as the comparison (Ujilestari et al., 2018).

2.5 Determination of Half-Maximal Lethal Concentration (LC_{50} value)

The toxicity in zebrafish was evaluated for 96 hours in each test group. Subjects were exposed to five concentrations of NSOE SNEDDS (500, 250, 125, 62.5, and 31.5 part per million (ppm)) and five concentrations of NSOE non-SNEDDS (125, 62.5, 31.25, 15.625, and 7.8125 ppm). One subject group

was given a combination of cosurfactants-surfactants to ensure that the use of surfactants and cosurfactants was not toxic to zebrafish. Each observation was performed in three replications. Zebrafish mortality was characterized by the absence of movement and tail response when touched. Dead fish were removed each day from the test aquarium, and their mortality was calculated (The Organization for Economic Co-operation and Development [OECD], 2018). The half-maximal lethal concentration (lethal concentration 50% or LC_{50}) value was the concentrations of NSOE SNEDDS and non-SNEDDS (ppm) that could kill 50% of the zebrafish in each test group. The LC_{50} values were calculated from the equation for the linear regression line between the concentration versus the percent mortality of zebrafish. After 96 hours, the mortality in each group was calculated in percent.

2.6 Statistical Analyses

The death percentage per concentration for each formulation was analyzed using the probit analysis (SPSS software version 21) to determine the LC_{50} values. The average of IC_{50} values of NSOE SNEDDS and non SNEDDS were compared using t-test and p value < 0.05 was considered as significant.

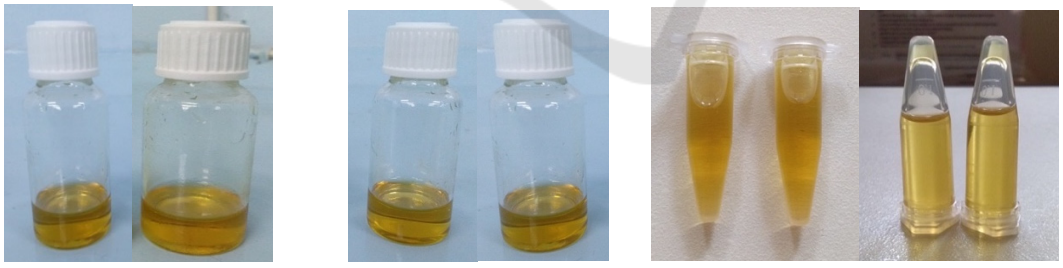
3 RESULTS.

Preparation of NSOE and NSOE SNEDDS Formulation: The extraction process produced NSOE, which was then formulated into a SNEDDS preparation. The procedure resulted in a water-soluble SNEDDS preparation characterized by a clear, transparent, slightly misty solution.

Formulation Stability Test: The three stability tests indicated that the NSOE SNEDDS had good physical stability, characterized by no organoleptic changes before or after the test, no phase separation, and no formation of crystal or sediment (Figure 1 and Table 1).

Globule Size, Zeta Potential, PDI, and %Transmittance: The particle size was in the 50–200 nm range, and the potential zeta value was lower than −30 mV, demonstrating the good stability of the nanoemulsion of NSOE. The PDI value was 0.499 and %transmittance was 64.414%, indicating that the SNEDDS preparation was not fully monodispersed (Table 2).

LC_{50} Value of Formulations: The toxicity of the test compound was calculated from the zebrafish mortality rate for 96 hours of treatment (Table 3). Combinations of the surfactant-cosurfactant (SCS) showed no subject deaths in all three replications. This indicated that using surfactants-cosurfactants in preparing NSOE SNEDDS was safe and nontoxic to zebrafish. The LC_{50} values for the NSOE SNEDDS and non-SNEDDS formulations calculated using a probit analysis are listed in Table 4. As shown in Table 4, although not statistically significant (p value > 0.05), the toxicity of the NSOE SNEDDS formulation was lower than in the non-SNEDDS. Therefore, the toxicities of NSOE SNEDDS and non-SNEDDS formulations were comparable.



(a) (b) (c)

Figure 1: Appearance of NSOE SNEDDS formulation (left, before the test; right, after the test); (a) before and after the heating-stability test; (b) before and after the freeze-thaw test; and (c) before and after the centrifugation test.

Table 1: Organoleptic appearance, phase separation, and precipitation before and after stability tests.

	Before the test	After the test		
	Organoleptic appearance	Organoleptic appearance	Phase separation	Precipitation
Heating-stability test	Brownish-yellow, clear, smelling typical of *N.sativa* L. oil	Brownish-yellow, clear, smelling typical of *N.sativa* L. oil	None	None
Freeze-thaw test				
Centrifugation test				

Table 2: Particle size, zeta potential, PDI, and %transmittance of NSOE SNEDDS formulation.

Particle size (nm)	Zeta potential (mV)	PDI	%Transmittance (%)
139.2	–59.5	0.499	64.414

Table 3: Deaths of zebrafish in a 96-hour observation of each test group.

Compound	Concentration	Number of subjects	Number of deaths			Mortality (%)			Mortality average (%)
			R1	R2	R3	R1	R2	R3	
NSOE SNEDDS	S1 (500 ppm)	7	7	7	7	100	100	100	100
	S2 (250 ppm)	7	4	7	7	57	100	100	86
	S3 (125 ppm)	7	0	3	7	0	42	100	47
	S4 (62.5 ppm)	7	1	1	0	14	14	0	9
	S5 (31.5 ppm)	7	0	1	0	0	14	0	5
NSOE non-SNEDDS	NS1 (125 ppm)	7	7	7	7	100	100	100	100
	NS 2 (62.5 ppm)	7	0	5	0	0	71	71	47
	NS 3 (31.25 ppm)	7	0	0	0	0	0	0	0
	NS 4 (15.625 ppm)	7	2	0	3	29	0	43	24
	NS 5 (7.8125 ppm)	7	0	0	0	0	0	0	0
SCS (control)	125 ppm	7	0	0	0	0	0	0	0

Abbreviations:
S1–S5, SNEDDS concentration 1–5
NS1–NS5, non-SNEDDS concentration 1–5
SCS, surfactant-cosurfactant
R1, replication 1; R2, replication 2; R3, replication 3

Table 4: LC_{50} values of NSOE SNEDDS and non-SNEDDS formulations.

Compound	Replication	LC_{50} value* (ppm)	Average $LC_{50} \pm$ SD (ppm)	p *value* **
NSOE SNEDDS	1	237.227	154.637 ± 75.609	0.138
	2	137.860		
	3	88.826		
NSOE non-SNEDDS	1	84.037	72.358 ± 15.253	
	2	55.101		
	3	77.936		

Note:
* LC_{50} values were obtained from the mortality percentage data at five concentrations of NSOE SNEDDS and non-SNEDDS formulations and analyzed using a probit analysis to obtain concentrations that caused the death of 50% of subjects.
** *p value* was significant at 0.05

4 DISCUSSIONS

This study investigated the toxicity of NSOE SNEDDS against adult zebra fish compared to the non SNEDDS. The results showed that the toxicity of NSOE SNEDDS lower than non SNEDDS, however, this difference was non statistically significant.

Surfactant selection is a crucial part in the preparation of SNEDDS formulations. Surfactants play an important role in forming nanoemulsions and reducing the surface tension between the two phases

(oil and water) for good emulsion dispersal (Patel et al., 2011). Surfactants also stabilize nanoemulsion preparations by maintaining the physical properties of the preparation and preventing damage to the bioactive compounds during processing and storage (Chuacharoen et al., 2019). Tween 80 is an n-hexane/water emulsion with non-ionic, nontoxic, and biocompatible properties that result in a low level of toxicity, making it safe to use. Tween 80 surfactant is widely used in the processing of nanoemulsion preparations in the pharmaceutical industry (Prieto & Calvo, 2013).

The addition of cosurfactants helps to reduce the size of nanoemulsion globules compared to the use of surfactants alone. PEG 400 is a widely used polymer cosurfactant in drug formulations. Its strong hydration property allows it to form a stronger interaction between the polymer and water compared to the polymer-polymer interaction, thereby increasing the emulsion mucoadhesion (Chen et al., 2019). The low toxicity of the Tween 80 and PEG 400 combination has been demonstrated in this study, characterized by the absence of death (mortality) in this group.

Successful nanoemulsion preparations are marked by a clear, transparent or slightly foggy appearance (Handayani et al., 2019), and good physical stability. Physical stability tests of the SNEDDS formulation of NSOE demonstrated good stability and fulfilled the requirements of the nanoemulsion (Senapati et al., 2016). The presence of a surfactant and cosurfactant reduced the surface tension between the oil and water phases of the emulsion. The greater the reduction of surface tension, the more stable the nanoemulsion formulation (Villar et al., 2012).

The mean globule size of the NSOE SNEDDS formulation was 139.2 nm, which meets the requirements for nanoemulsion particle size in the drug delivery system (50–200 nm), making it suitable for use in both food and drug industries (McClements & Rao, 2011). The smaller the active substance particle size in a nanoemulsion formulation, the better the stability and distribution in dissolution media. The zeta potential is a parameter for estimating surface loads to understand the physical stability of preparations and is important in characterizing nanoemulsion preparations. The zeta potential value of the NSOE SNEDDS formulation was −59.5 mV, which met the criterion of a stable nanoparticle of more than +30 mV or smaller than −30 mV (Kumar & Dixit, 2017).

The PDI value was 0.499, indicating that the particles formed in SNEDDS formulations were not fully monodispersed. The PDI parameter indicates the globule size distribution; the lower the PDI value, the b etter the level of monodispersity. In essence, PDI is a dimensionless particle heterogeneity index. A PDI of <0.3 meets the monodispersion criterion (Danaei et al., 2018). Efforts to reduce PDI include prolonging the ultrasonication process up to 30 minutes (Mahbubul, 2019). Although the PDI value in this study was more than 0.3, this was fairly good because the particle size distribution of a SNEDDS formulation is deemed heterogeneous (polydispersed particles) if PDI exceeds 0.7 (Danaei et al., 2018).

The value of %transmittance indicates the level of clarity of SNEDDS preparations; the closer the %transmittance to 100%, the smaller the particle size in nanometers, and the closer the optical clarity is to water (Khan et al., 2015). SNEDDS preparations with nearly 100% transmittance appear clearer and more transparent with a greater possibility of absorption in the digestive tract (Yen et al., 2017). In this study, the %transmittance was only 64%, indicating that the SNEDDS formulation of NSOE was of poor quality. The %transmittance may be improved by increasing the surfactant concentrations. A previous study showed that raising surfactant concentrations (Tween 80) in a SNEDDS preparation containing vitamin D led to a greatly reduced globular size and an increased oil-water interface. However, as the surfactant concentration continues to increase, the globule size also becomes larger. Thus, to produce the optimal globule size, the ideal ratio of surfactant/oil is 1:1 (Guttoff et al., 2015). In a different study, an increased surfactant concentration in a SNEDDS formulation containing curcumin increased %transmittance to 92.86%–99.51% (Chuacharoen et al., 2019). These findings suggest that higher surfactant concentrations improve the trapping of active compounds in the particles, leading to an increase in %transmittance to almost 100%.

The acute toxicity test is designed to determine the toxic effects of a particular dose in a short time. Usually, acute toxicity is observed from 24 hours up to 7 days. Such tests aim to evaluate adverse effects on a test organism after substance exposure within 24 hours by oral, skin, or inhalation routes (Saganuwan, 2017). The test uses the LC_{50} to indicate toxicity, which is determined based on the mortality ratio of experimental animals (Parasuraman, 2011). The use of adult zebrafish to test the toxicity of a drug candidate has currently replaced the use of mammals to implement the principles of reduction, replacement, and refinement in the use of animals for research. Several studies have shown that zebrafish in both the embryonic and adult forms have an equal

sensitiity to chemicals, which is indicated by the slight difference in their LC_{50} values (Kovrižnych et al., 2013). Embryonic and adult zebrafish also have comparable sensitivity to cationic and nonionic surfactants (Vaughan & Van Egmond, 2010).

The toxicity tests of the SNEDDS and non-SNEDDS formulations of NSOE on zebrafish were conducted for 96 hours in each test group. For the SNEDDS formulation, 100% zebrafish mortality occurred at 500 ppm concentration; at 31.5 ppm, the zebrafish mortality was zero. As for the non-SNEDDS group, 100% zebrafish mortality occurred at 125 ppm concentration and no deaths occurred at 7.8125 ppm. The probit analysis revealed that the LC_{50} value of the SNEDDS formulation of NSOE was 154.637 $\pm$ 75.609 ppm, whereas the LC_{50} of the non-SNEDDS formulation was 72.358 $\pm$ 15.253 ppm. These LC_{50} values showed that the SNEDDS preparation of NSOE was less toxic than the non-SNEDDS preparation; however, this difference was not significant ($p = 0.138$).

This result is in agreement with previous studies that showed that SNEDDS formulations were safer than their non-SNEDDS preparations. The toxicity of a SNEDDS formulation of *Ipomoea reptans* against Vero cells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay did not cause Vero cell death; instead, the cell culture growth improved (Chabib et al., 2019). Another study showed that the preparation of arteether SNEDDS as an antimalarial in *Plasmodium yoelii nigeriensis*-infected mice showed a better bioavailability with minimal toxicity (Dwivedi et al., 2014). In another acute toxicity test, the SNEDDS formulation of bay leaf chloroform extract also gave a very high LC_{50} value; in the subchronic toxicity test, the preparation had minimal effects on the pancreas, kidneys, and liver at low to moderate doses. However, organ damage was directly proportional to the increasing dosage (Prihapsara et al., 2018).

This study results might have implications for nanoparticle research and might recommend against the use of best combinations of surfactant and co-surfactant in preparation of SNEDDS formulations. However, further ongoing research is required to ensure the safety of NSOE SNEDDS formulation for oral drug delivery in animals and modification of this formulation to guarantee their future application.

One limitation of this study was the composition of the SNEDDS formulation of NSOE, which was based on previous research. SNEDDS formulation preparation using this composition resulted in good stability as evidenced by the stability tests and measurements of globules and zeta potential, but its PDI and %transmittance was not optimal. Another limitation is that only the toxicity was tested, not the efficacy or pharmacological activity. The SNEDDS formulation of NSOE is nontoxic, but the efficacy remains to be determined. Thus, an investigation into the efficacy and toxicity in one study is recommended.

5 CONCLUSIONS

The LC_{50} value of the SNEDDS formulation of NSOE (154.637 ± 75.609) ppm was better than its non-SNEDDS form (72.358 ± 15.253 ppm), but this difference was not statistically different. Thus, the toxicities of SNEDDS and non-SNEDDS formulations of NSOE were comparable. The SNEDDS preparation did not reach optimal conditions, as indicated by good globule size and zeta potential values but non-optimal PDI and %transmittance values. Thus, the toxicity of the SNEDDS formulation may improve with optimization.

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