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Exploring the Safety of the Sustainable Toxicity Testing in Zebrafish and Brine Shrimp Using Nanoemulsions Formulated from Fish Byproducts and Lemon Oil

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Abstract: Nanoemulsions, characterized by their nanosized particles ranging from 20 to 200 nm, are effective carriers for drug molecules. Our novel oil-in-water nanoemulsion, NE-FLO™, formulated from lemon and fish byproduct oils, demonstrates promising antioxidant and anti-inflammatory activities, with initial studies indicating nontoxicity to normal skin cells. This study investigated the safety of NE-FLO™ using brine shrimp (*Artemia salina*) and zebrafish (*Danio rerio*) models, focusing on concentration-dependent effects and LC_{50} values. At lower concentrations (0.1 mg·L^{-1}, 0.01 mg·L^{-1}, and 0.001 mg·L^{-1}), NE-FLO™ showed minimal toxicity without adverse effects. However, at 1 mg·L^{-1}, reduced survival rates indicate potential toxicity. Specifically, this concentration also induces altered swimming behaviors in zebrafish. LC_{50} values are 8.7474 mg·L^{-1} for brine shrimp and 0.316 mg·L^{-1} for adult zebrafish. These results underscore the necessity for further detailed investigations into NE-FLO™, balancing its therapeutic benefits with potential toxicity risks. This study emphasizes the importance of optimizing nanoemulsion formulations from fish oil and conducting comprehensive safety assessments to meet regulatory standards.

Keywords: nanoemulsion; zebrafish; toxicity; sustainability; fish oil; safety

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1. Introduction

Nanoemulsion technology has garnered significant attention for its diverse applications and potential in promoting sustainable practices. Fish oil, renowned for its health benefits, faces challenges due to its hydrophobic nature and poor bioavailability. To overcome these limitations, fish oil nanoemulsions—nanoscale droplets of fish oil dispersed in water—have been developed. These nanoemulsions have shown promise as effective delivery systems for omega-3 fatty acids [1,2]. Despite their potential, concerns persist regarding potential toxicity at higher concentrations [3].

Various nanoemulsions, such as those loaded with soybean isoflavone aglycones-rich fraction and essential clove oil, have demonstrated wound-healing properties [4]. However, nanoemulsions containing fish oil, despite their reported anti-inflammatory and antibacterial effects, remain relatively underexplored [5]. Recent studies highlight the potential of nanoemulsions as carriers for bioactive compounds, enhancing their stability and bioavailability.

Our previous research has demonstrated the antibacterial and anti-inflammatory potential of NE-FLO™ [5]. Zebrafish, which share a 70% genomic similarity with humans [6], serve as an ideal model for toxicity testing, as depicted in Figure 1. Studies indicate that exposure to high dosages of fish oil nanoemulsions can induce oxidative stress and histopathological changes in zebrafish [7,8]. Notably, one study found that nanoemulsions containing lemon oil had minimal adverse effects on Mozambique tilapia development and behavior, suggesting their suitability for eco-friendly toxicity assessments [9]. Additionally, Swathy et al. [10] examined the effects of black pepper oil nanoemulsion on shrimp. Their study reported no significant mortality or developmental abnormalities, highlighting the potential of these nanoemulsions as a non-toxic testing medium. Brine shrimp provides a simpler and cost-effective model for preliminary toxicity screening, which is valuable in the early-stage testing of new substances.

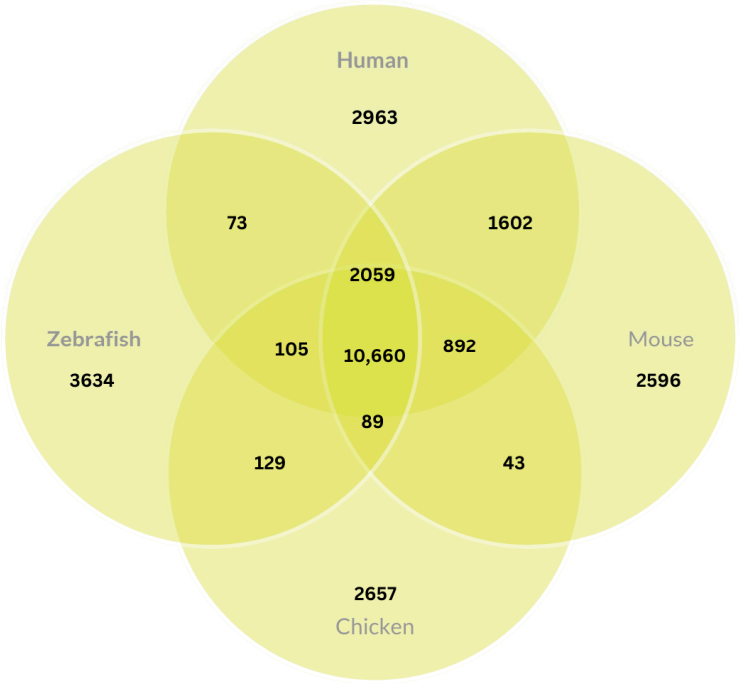

Figure 1. Orthologue genes shared between the zebrafish, human, mouse and chicken genomes, using orthology relationships from Ensembl Compara 63. Genes shared across species are considered in terms of copies at the time of the split. For example, a gene that exists in one copy in zebrafish but has been duplicated in the human lineage will be counted as only one shared gene in the overlap s reproduced from Howe et al. [6] Published by Nature, 2013, under Creative Commons Attribution-Non-Commercial-Share Alike license (http://creativecommons.org/licenses/by-nc-sa/3.0/).

The adult zebrafish toxicity test, known for its cost-effectiveness, reproducibility, and high throughput capacity, serves as a reliable alternative to traditional animal testing methods [11]. Zebrafish have been extensively used in toxicological research, providing valuable insights into chemical toxicity and aiding drug development [12,13].

Incorporating fish byproducts in the formulation of nanoemulsions provides a sustainable approach to waste reduction and adds value to fisheries. The effectiveness of fish oil-derived emulsions in delivering hydrophobic compounds without significant cellular toxicity has been reported [6]. This approach not only addresses waste management but also promotes the use of renewable resources to develop eco-friendly products. Therefore, this study aims to assess the toxicity profile of the NE-FLO™ optimized formulation to balance beneficial properties with minimal toxicity. This research contributes to the field of nanoemulsion science and holds practical implications for the pharmaceutical, nutraceutical, and food industries. Enhanced bioavailability of omega-3 fatty acids in these sectors can lead to better health outcomes and address dietary deficiencies on a larger scale.

2. Materials and Methods

2.1. Materials

This study used specific materials and equipment for experimental procedures. Brine shrimp cultivation involved *Artemia salina* eggs from Ocean Nutrition and pure *Artemia* cysts sourced from the Great Salt Lake, supplemented with sodium chloride (Sigma–Aldrich, catalog number: S7653, St. Louis, MO, USA) and sea salt (Tropic Marin, Hünenberg, Switzerland) in seawater conditions. Zebrafish (*Danio rerio*) maintenance utilized adult fish aged between 3 and 6 months from the Universiti Putra Malaysia (UPM) *Danio rerio* laboratory, housed in four 3 L fish tanks. Water quality was maintained using a Panasonic TK-CS20 water filter (Kadoma, Japan), supplemented with commercial fish feed Otohime B1 (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan), water conditioner using Nutrafin aqua plus (RC Hagen Ltd., Montreal, QC, Canada) and Nutrafin Clear (RC Hagen Ltd., Montreal, QC, Canada). Each tank was equipped with aquaria biochemical sponge foam filters to maintain water quality, and water parameters were monitored using an API freshwater master test kit. Other materials and consumables were used as per standard laboratory practices.

2.2. Ethical Clearance

Our research protocol was reviewed by the Institutional Animal Care and Use Committee (IACUC) and approved for animal use under the ID IACUC 2023-001 on 15 April 2023, valid until 15 April 2024. Animal experiments were completed in August 2023. Proper disposal procedures for zebrafish carcasses, as outlined in the IACUC protocol, were followed [14,15].

2.3. Preparation of the Nanoemulsion of Fish Byproducts and Lemon Oils (NE-FLO)™

The ultrasonication method utilized in this study was adapted from Azmi et al. [5] and Espinosa-Andrews and Paez-Hernandez [16]. The preparation of fish byproducts and lemon oil nanoemulsion (NE-FLO)™ involved a two-step process. First, the aqueous phase was created by stirring Tween 80 and the cosurfactant in pure distilled water for 15 min to form the continuous phase. Simultaneously, the oil phase, consisting of fish oil and lemon oil, was stirred at 500 rpm until thoroughly mixed (specific data are withheld due to patent processing) using a magnetic stirrer. Next, the oil phase was added dropwise into the continuous aqueous phase while maintaining the stirring speed. The mixture was homogenized using a T25 digital Ultra-Turrax homogenizer (Staufen, Germany) at 20,000 rpm for 3 min, resulting in a coarse oil-in-water emulsion. To achieve nanosized droplets, the coarse emulsion underwent ultrasonication for 42 min, an optimized duration for this study at 80% of the maximum output of 100 W. Following ultrasonication, the solution was tested to verify the formation of nanosized droplets. Figure 2 shows the process of preparing the nanoemulsion [5].

2.4. Properties of Nanoemulsion of Fish Byproducts and Lemon Oils (NE-FLO™)

The particle size, polydispersity index (PDI), and zeta potential of NE-FLO™ were measured using a particle size analyzer (Malvern Instruments, Malvern, UK) at 25 °C. The samples were diluted with deionized water at a 1:9 ratio to prevent backscattering. The refractive indices of the particles and water used were 1.54 and 1.33, respectively. Each measurement was performed three times [5].

2.5. Diluting NE-FLO™ for Toxicity on Brine Shrimp

NE-FLO™ was stored in the laboratory at 4 °C until the commencement of the experimental studies. Stock solutions of NE-FLO™ were prepared by diluting the NE-FLO™ samples with seawater to achieve six different concentrations: 10 $mg \cdot L^{-1}$, 1 $mg \cdot L^{-1}$, 0.1 $mg \cdot L^{-1}$, 0.01 $mg \cdot L^{-1}$, 0.005 $mg \cdot L^{-1}$, and 0.001 $mg \cdot L^{-1}$. The contents were vigorously vortexed for 20 s at 2000 rpm to ensure a uniform distribution. The initial dilution sample, referred to as 'stock 0', was prepared by mixing 0.10 g of NE-FLO™ in 10 mL of seawater

with 1 mL of Tween 80% in 90 mL of seawater. Appropriate volumes of the stock solutions were then promptly transferred into the exposure containers containing *Artemia* in seawater. Figure 3 illustrates the steps required to prepare the brine shrimp for toxicity testing.

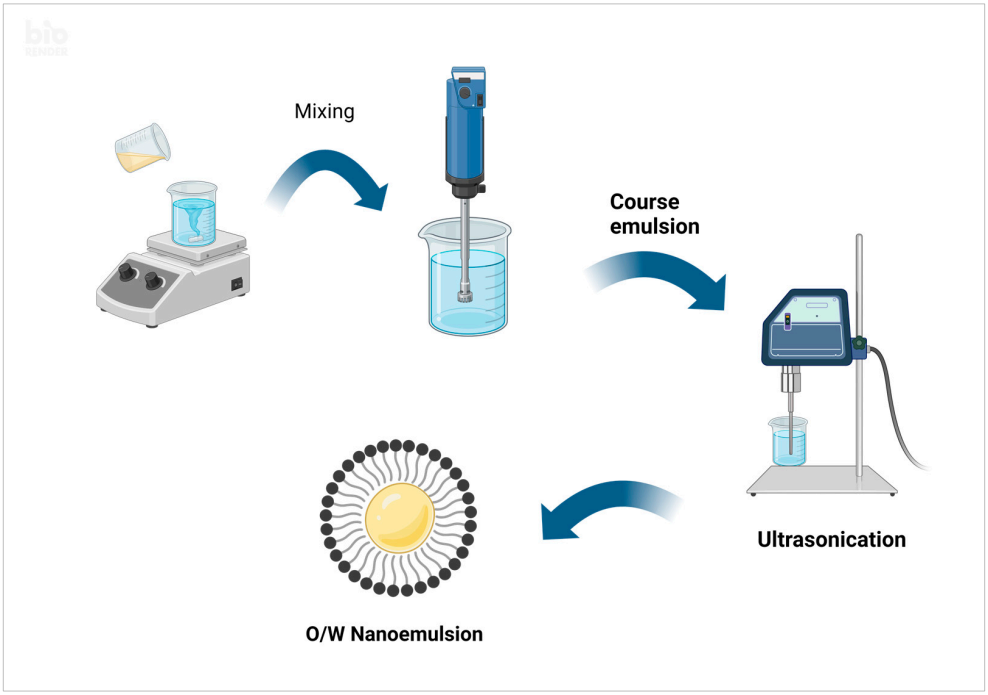

Figure 2. Schematic diagram of the preparation of NE-FLO™ using the ultrasonication method. The process involves the emulsification of lemon oil and fish byproduct oil, followed by ultrasonication to form a stable nanoemulsion. Reproduced with permission from Azmi et al. [5], Molecules; published by MDPI, 2022 under Creative Commons Attribution 4.0 International.

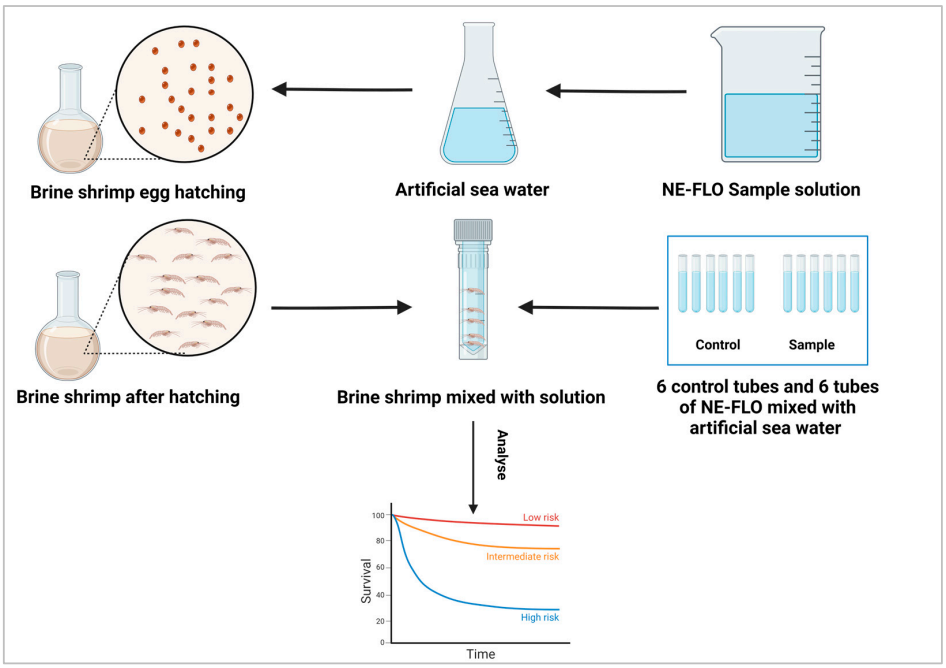

Figure 3. Preparation of the brine shrimp assay. Brine shrimp eggs hatch in artificial seawater over 24 h. They were then placed in various concentrations of NE-FLO™ and control samples to measure mortality rates.

2.6. NE-FLO™ Dilution for Adult Zebrafish

Stock solutions were prepared by diluting the nanoemulsion sample with filtered tap water to achieve concentrations of 1 mg·L^{-1}, 0.1 mg·L^{-1}, 0.01 mg·L^{-1}, and 0.001 mg·L^{-1}. These mixtures were vortexed for 20 s at 2000 rpm to ensure a uniform distribution. For instance, to create a 0.01 mg·L^{-1} concentration, a 5-L tank of clean fish tank water was prepared. The required volume of nanoemulsion treatment was calculated by using Equation (1).

$$\text{Volume} = \frac{\text{Desired concentration } \left(\text{mg·mL}^{-1}\right) \times \text{Total tank volume (liters)}}{\text{Concentration of nanoemulsion treatment } \left(\text{mg·mL}^{-1}\right)} \quad (1)$$

The calculated volume was then added to the tank and gently mixed to ensure thorough dispersion. Following this dilution process, the fish in the tank were observed for behavioral and health changes. The experimental protocol adhered to the Organization for Economic Cooperation and Development (OECD) guidelines for chemical testing [17].

2.7. Preparation of Artemia salina Larvae (Brine Shrimp)

The brine shrimp eggs (*Artemia salina*) and pure *Artemia* cysts were sourced from the Great Salt Lake. Geographical variations do not affect the assay. The cysts were prepared in-house and stored in a refrigerator at 4 °C. The incubated larvae hatched in artificial seawater (3% *m*/*v*) made by dissolving a suitable amount of Instant Ocean® salt (Blacksburg, VA, USA) in deionized water, stirring it for 24 h with aeration, and filtering it through Millipore cellulose filters. *Artemia* was hatched using a method described by Zhu B et al. [18], with some modifications. In brief, encysted *Artemia* was hydrated in distilled water for 12 h at 4 °C before being rinsed to remove floating cysts. Sinking cysts were collected using a Buchner funnel and rinsed in cold deionized water. Precleaned cysts (3 g) were incubated at 30 °C in 1.5 L saltwater in a conical plastic container with graduations. A fluorescent bulb provided continuous daylight at 60 W. An aquarium air pump supplied aeration through a hose flowing to the bottom of the hatching device. Under these conditions, *Artemia* larvae hatched within 24 h.

2.8. Preparation of Adult Zebrafish

Zebrafish were supplied by the UPM zebrafish laboratory and delivered to the desired location (INHART Laboratory @ KICT Building, International Islamic University Malaysia, Gombak campus). They were acclimatized to their new environment following the acclimatization protocol for three weeks [15,19]. The number of fish lost during acclimatization was about 10% of the total number of fish. The adult zebrafish, both male and female, aged between 3 and 6 months and weighing 0.45 ± 0.05 g, were exposed to the solution with and without the fish oil nanoemulsions in covered 5 L plastic tanks [20,21]. Each experimental and control group contained five fish, with a ratio of 1 g of fish weight to 1.8–2.0 L of water. All tank water parameters, including pH, high range pH, ammonia, nitrite, and nitrate, were kept stable to ensure scientific accuracy. Control groups were maintained in water at 25 ± 1 °C without the nanoemulsion treatment. The total hardness of the water was 13 N° dgH, and the light-dark regimen was 12 h of light and 12 h of darkness. The oxygen concentration was maintained above 60%, and the pH was kept between 6.5 and 7.0. The test was initiated by diluting the four concentrations of nanoemulsion (1 mg·L^{-1}, 0.1 mg·L^{-1}, 0.01 mg·L^{-1}, and 0.001 mg·L^{-1}) with fish tank water. A negative control tank, which received the same care except for the nanoemulsion treatment, was used to observe any changes or effects. Once the pH and other parameters (chlorine, nitrite, nitrate, ammonia) were tested and adjusted, five adult zebrafish were added to each tank. The fish were monitored every 24 h for three days for any signs of discomfort, illness, rejection, or mortality. The fish were fed 4% of body weight in food per day, as specified in the zebrafish care and maintenance protocol [22,23].

2.9. Experiment Setup for Artemia Larvae

Acute toxicity testing was conducted according to the guidelines established by OECD [17]. *Artemia* larvae were exposed to six different concentrations of NE-FLO™ for 24 h: 10 mg·L^{-1}, 1.0 mg·L^{-1}, 0.1 mg·L^{-1}, 0.01 mg·L^{-1}, 0.005 mg·L^{-1}, and 0.001 mg·L^{-1}, following OECD protocols. A positive control group (10 mg of tetracycline in 1 mL of seawater) without the test substance was also included. Exposures were conducted at 24 ± 2 °C in triplicate using 1.0 L conical plastic containers filled with 500 mL of saltwater, maintaining a total salinity of 2.9–3% *m*/*v* and a light regime of 16:8 h light: dark. No food was provided during exposure. Detailed experimental conditions, percentages, and log concentrations are presented in Table 1 and Figure 3. Following exposure, a portion of each group's *Artemia* was rinsed with seawater and immediately transferred to a clean seawater bath. All tests were conducted in duplicate to ensure the accuracy of results.

Table 1. Main properties of NE-FLO™.

Measurement	NE-FLO Nanoemulsion Value
Mean Particle Size (Z-averages)	44.40 ± 0.11 nm
Polydispersity Index (PDI)	0.077
Zeta Potential	−5.02 ± 0.22 mV
pH	4.27 ± 0.01

2.10. Experiment Design for Adult Zebrafish

A controlled laboratory environment was established for the adult zebrafish toxicity test with four separate fish tanks, each accommodating a group of five zebrafish. Each tank was subjected to a distinct concentration of the nanoemulsion treatment, while one tank served as the control with no additional substances introduced. These specific concentrations were determined based on the results of the brine shrimp toxicity test, taking into account the observed number of fatalities. Throughout the testing period, the zebrafish were fed a diet of commercial fish food, specifically Otohime B1 (Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan), to meet their nutritional requirements. Environmental conditions were meticulously maintained, with temperature levels ranging between 26 and 28 °C and pH levels maintained within the range of 6.8 to 7.5. Figure 4 illustrates the laboratory setup employed during the adult zebrafish toxicity test.

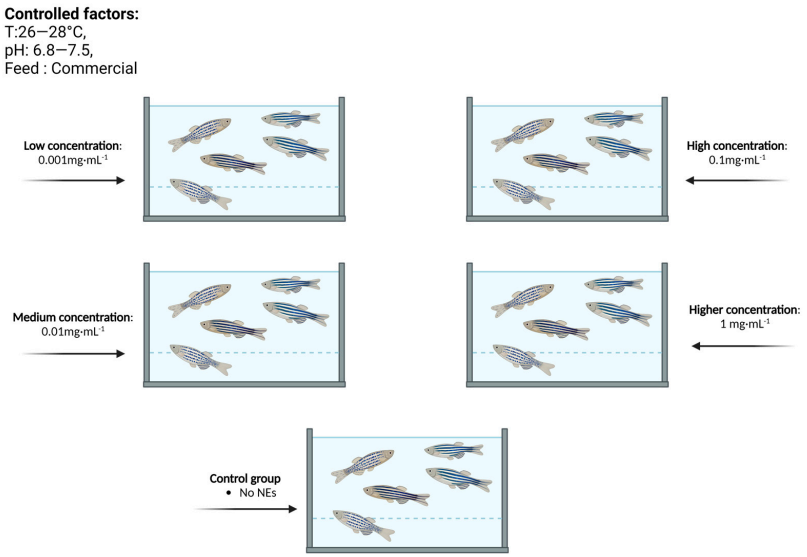

Figure 4. Illustration of the laboratory setup for the adult zebrafish toxicity test, displaying the arrangement of fish tanks. The tested concentrations, determined based on the brine shrimp test results, include low (0.001 mg·L^{-1}), medium (0.01 mg·L^{-1}), high (0.1 mg·L^{-1}), and a higher concentration (1 mg·L^{-1}) alongside a control tank. [NOTE: 1 ppm = 1 mg·L^{-1}].

2.11. Calculation of LC_{50}

The LC_{50} value for NE-FLO™ was determined using logistic regression analysis. Various concentrations of NE-FLO™ in mg/L were tested, and the mortality rates were recorded. The concentrations were log-transformed, and the mortality data were converted to proportions. A logistic regression model was then fitted to the data, with the log-transformed concentrations as the predictor variable. The LC_{50} value, representing the concentration at which 50% mortality is expected, was calculated from the model's parameters. The analysis was performed using the 'statsmodels' library in Python 3.11.10 (2024).

2.12. Statistical Analysis

In this study, statistical analyses were carried out using Microsoft Excel (Microsoft 365, version 2308).

2.13. OpenAI Utilization

In this study, a Python-based method was employed to calculate the LC_{50} value for NE-FLO™. Logistic regression analysis was performed on log-transformed concentrations and corresponding mortality data, with model fitting carried out using the stats models library in Python 3.11.10 (2024). ChatGPT was used to assist in developing and optimizing the code for the LC50 determination. Additionally, ChatGPT was utilized to edit and check the manuscript's English language for coherence and clarity. All outputs generated by ChatGPT were thoroughly reviewed and verified by the authors to ensure accuracy.

3. Results and Discussion

3.1. Properties of the Nanoemulsion of Fish Byproducts and Lemon Oils (NE-FLO™)

Table 1 shows the primary quantitative results of the NE-FLO nanoemulsion investigation. The average particle size (Z-averages) is 44.40 ± 0.11 nm, indicating a highly fine distribution of particles within the nanoemulsion. A low PDI value of 0.077 reflects a high consistency and uniformity of the nanoemulsion droplet size. The zeta potential recorded at -5.02 ± 0.22 mV indicates adequate electrostatic stabilization despite being below the ideal stability range (greater than $|30|$ mV), thereby mitigating any concerns of flocculation or sedimentation. At higher concentrations, instability may lead to aggregation, potentially increasing toxicity. Future studies should assess the stability of the nanoemulsion in brine water and evaluate the impact of any aggregation on toxicity. Lastly, the nanoemulsion's pH is 4.27 ± 0.01, being acidic, which may influence the zeta potential and overall formulation stability [5].

3.2. Artemia Larvae Toxicity Test Observations

The toxicity experiments were conducted in triplicates on brine shrimp. Each trial involved six different concentrations of brine shrimp media and fish oil nanoemulsions, as indicated in Table 2. The mortality rates are calculated according to the equation:

$$\text{Mortality } \% \ = \ \frac{\text{Shrimp No.(initial)}}{\text{Total Mortality}} \ \times \ 100. \tag{2}$$

In the saltwater media with concentrations of 0.001 mg·L^{-1}, 0.005 mg·L^{-1}, and 0.01 mg·L^{-1}, the mortalities observed were five brine shrimps or lower. However, at higher concentrations (0.1 mg·L^{-1}, 1 mg·L^{-1}, and 10 mg·L^{-1}), a consistent mortality rate of 100.0% was observed, indicating that all the brine shrimps in these concentrations died. This suggests that the brine shrimp could not survive at higher concentrations, indicating these levels are highly toxic. The increase in mortality at higher concentrations highlights the critical impact of dosage on brine shrimp viability.

Table 2. Results of the acute toxicity test on *Artemia* Larvae.

	Sample Conc. ($mg \cdot L^{-1}$)	Log Conc. ($mg \cdot L^{-1}$)	Replicate One				Replicate Two				Replicate Three			
			Shrimps No. (Initial)	Shrimps No. (Final)	Total Mortality	Mortality %	Shrimps No. (Initial)	Shrimps No. (Final)	Total Mortality	Mortality %	Shrimps No. (Initial)	Shrimps No. (Final)	Total Mortality	Mortality %
Positive Control *	0.001	−2.3	10	7	3	30.0%	10	7	3	30.0%	10	7	3	30.0%
	0.005	−1.8	10	6	4	40.0%	10	6	4	40.0%	10	6	6	60.0%
	0.01	−1.5	10	5	5	50.0%	10	5	5	50.0%	10	5	5	50.0%
	0.1	−0.8	10	0	10	100.0%	10	0	10	100.0%	10	0	10	100.0%
	1	0	10	0	10	100.0%	10	0	10	100.0%	10	0	10	100.0%
	10	0.8	10	0	10	100.0%	10	0	10	100.0%	10	0	10	100.0%
Sample **	0.001	−2.3	10	8	2	20.0%	10	8	2	20.0%	10	8	2	20.0%
	0.005	−1.8	10	7	3	30.0%	10	7	3	30.0%	10	7	3	30.0%
	0.01	−1.5	10	7	3	30.0%	10	6	4	40.0%	10	7	3	30.0%
	0.1	−0.8	10	6	4	40.0%	10	6	4	40.0%	10	6	4	40.0%
	1	0	10	5	5	50.0%	10	4	6	60.0%	10	6	4	40.0%
	10	0.8	10	4	6	60.0%	10	3	7	70.0%	10	4	6	60.0%
Negative Control * (Artificial Seawater)**	**Replicate 1**		10	9	1		10							
	Replicate 2		10	8	2		20							
	Replicate 3		10	9	1		10							
	Average total death			1.33										

* Positive control: Diluted tetracycline in seawater added to the brine shrimp with concentrations of 0.001 $mg \cdot L^{-1}$, 0.005 $mg \cdot L^{-1}$, 0.01 $mg \cdot L^{-1}$, 0.1 $mg \cdot L^{-1}$, 1 $mg \cdot L^{-1}$, and 10 $mg \cdot L^{-1}$. ** Sample: Diluted NE-FLO™ treatment added to the brine shrimp with concentrations of 0.001 $mg \cdot L^{-1}$, 0.005 $mg \cdot L^{-1}$, 0.01 $mg \cdot L^{-1}$, 0.1 $mg \cdot L^{-1}$, 1 $mg \cdot L^{-1}$, and 10 $mg \cdot L^{-1}$. *** Negative control: Artificial seawater as a medium for the brine shrimp.

Similarly, mortality rates were calculated for the NE-FLO™ treatments at the same concentrations. It was observed that the concentrations of 0.001 mg·L^{-1}, 0.005 mg·L^{-1}, and 0.01 mg·L^{-1} resulted in 2 to 3 brine shrimp mortalities, while the concentrations of 0.1 mg·L^{-1}, 1 mg·L^{-1}, and 10 mg·L^{-1} resulted in 4 to 6 mortalities. Specifically, the concentration of 0.1 mg·L^{-1} exhibited the lowest mortality rate at 40.0%. The concentration of 1 mg·L^{-1} had a mortality rate of 50.0%. Lastly, the concentration of 10 mg·L^{-1} exhibited a 60.0% mortality rate. These percentages reflect the proportion of mortalities relative to the total number of samples tested at each concentration.

At the 48 h mark, live brine shrimp counts were recorded again, and the mortality rates for each concentration remained consistent with the first trial except for a slight rise in the mortality rates for the concentrations of 1 mg·L^{-1} and 10 mg·L^{-1} where the mortality rates are observed to be 60% and 70%, respectively. After 72 h, it was observed that higher concentrations of diluted tetracycline in seawater (0.1 mg·L^{-1}, 1 mg·L^{-1}, and 10 mg·L^{-1}) resulted in a 100% mortality rate among the brine shrimps. In contrast, fish oil nanoemulsion treatments at equivalent concentrations showed lower mortality rates, ranging between 40.0% and 60.0%. Specifically, concentrations resulted in 3 to 4 mortalities, signifying a comparatively higher survival rate compared to the saltwater media concentrations.

As observed from Table 2, the acute toxicity test results for brine shrimp across various concentrations provide an understanding of the toxic effects of the tested sample. At lower concentrations, the sample showed relatively low toxicity to brine shrimp. However, as concentrations increased, a marked rise in mortality rates was observed. The control group at 0.005 mg·L^{-1} and 0.01 mg·L^{-1} exhibited mortality rates of 40% and 50%, respectively, 26.67%, and maintained consistency, indicating a threshold where adverse effects become noticeable. Interestingly, the sample group at 0.01 mg·L^{-1} displayed similar mortality rates, suggesting a comparable level of toxicity to the control at this concentration.

A significant increase in mortality is observed at 0.1 mg·L^{-1}, with both control and sample groups experiencing a 100% mortality rate, indicating a concentration-dependent response where brine shrimp are unable to survive higher doses. This finding highlights the concentration level at which the sample exhibits acute toxicity, consistent with the effects seen in the control substance. At the highest concentrations (1 mg·L^{-1} and 10 mg·L^{-1}), positive control and sample groups maintained high mortality rates at 100%. However, the sample group showed slight variation with mortality rates of 50% and 60%, respectively, indicating a possible variation in the lethal dose between replicates.

Such variability emphasizes the influence of environmental or procedural factors such as temperature, water quality, and contaminants, which can significantly impact the toxicity of substances to aquatic organisms. For instance, variations in water temperature and wastewater effluent compositions have been shown to affect the results of toxicity bioassays [24]. Hence, careful monitoring and maintenance of water quality during testing are crucial to minimize these variables. The negative control group treated with artificial seawater displayed significantly lower mortality rates, with an average total death of only 1.33. This stark contrast indicates that the observed mortalities in the other groups are directly attributable to the concentrations of the tested substances rather than environmental conditions.

Our results suggest that the fish oil nanoemulsion treatment exhibited relatively low mortality rates even at higher concentrations compared to the artificial seawater brine shrimp media and the positive control group. The selected concentrations for the zebrafish tests (0.1 mg·L^{-1}, 0.01 mg·L^{-1}, and 0.001 mg·L^{-1}) were chosen to account for potential differences in sensitivity between brine shrimp and larger organisms like zebrafish. Brine shrimp may respond differently to the tested concentrations compared to zebrafish [25]. To further assess these differences, higher concentrations (1 mg·L^{-1} and 10 mg·L^{-1}) were also tested on zebrafish, allowing for the observation of effects without reaching the high mortality rates seen at the maximum concentrations in the brine shrimp tests, which led to 100% mortality rates. Figure 5 illustrates the relationship between the NE-FLO™

sample's log concentration and the mortality rate. The drastic change in zebrafish livability observed between 0.1 mg·L^{-1} and 1 mg·L^{-1} of NE-FLO could be attributed to a limit effect, where a critical concentration is reached, causing significant physiological stress. At lower concentrations (e.g., 0.1 mg·L^{-1}), the nanoemulsion may not have reached a level sufficient to disrupt cellular processes or provoke a toxic response. However, at higher concentrations (e.g., 1 mg·L^{-1}), the accumulation of the nanoemulsion may surpass the organism's capacity to cope with stress, leading to mortality.

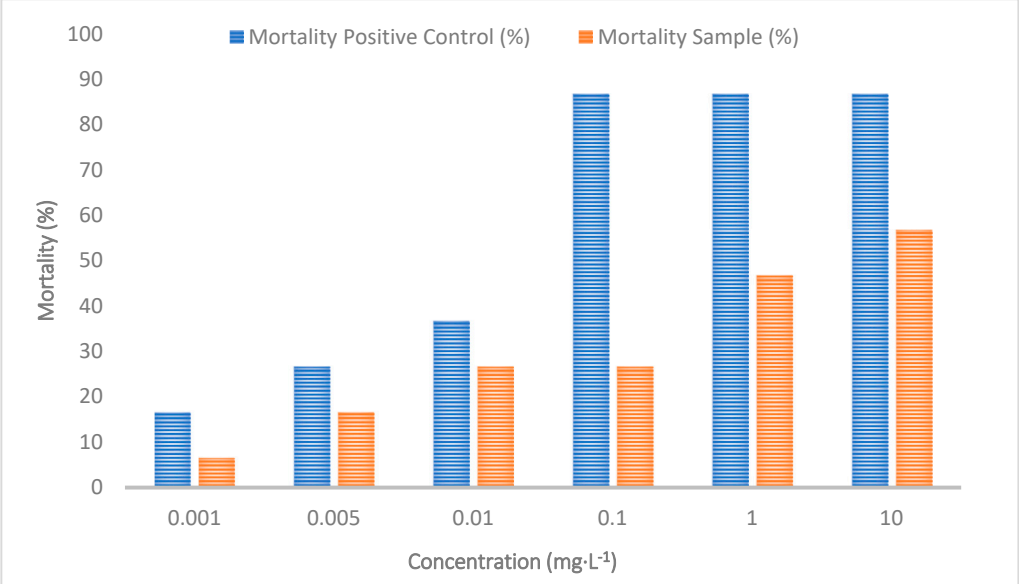

Figure 5. Effect of NE−FLO™ concentration on brine shrimp mortality percentage.

The logistic regression analysis estimated the LC_{50} value for the tested substance to be 8.7474 mg·L^{-1}, indicating moderate toxicity to brine shrimp. This value suggests that the substance poses a moderate risk to aquatic environments where brine shrimp are present. While the current model provides a useful initial estimate of the LC_{50}, further studies with larger sample sizes and a wider range of concentrations would enhance the robustness and precision of these findings. Consequently, it is advisable to exercise caution when using this substance in relevant environments to mitigate potential impacts on brine shrimp.

3.3. Adult Zebrafish Toxicity Test Observation

On day zero of the zebrafish acute toxicity test, no mortality was observed, and all the fish exhibited signs of good health. This was evident from their normal swimming patterns and responsive behavior towards external stimuli in all three tanks treated with fish oil nanoemulsion. The fish were fed according to recommended guidelines, and no decrease in appetite was observed. Additionally, their excretions appeared normal in color and quantity compared to the zebrafish observed in the negative control tank (Figure 6). In contrast, zebrafish exposed to higher concentrations of toxic substances often display abnormal swimming patterns, such as erratic or hyperactive swimming, circling or looping behavior, or even immobility. Loss of equilibrium, where fish swim on their sides or upside down, is another sign of severe distress. These abnormal behaviors have been well-documented as indicators of neurotoxicity or stress in zebrafish models of environmental toxicity. None of these abnormal behaviors were observed in the fish oil nanoemulsion treatment groups, further indicating the safety and nontoxicity of the sample at the tested concentrations [26].

After a 24 h period, the zebrafish were reevaluated. No mortality was recorded at concentrations of 0.1 mg·L^{-1}, 0.01 mg·L^{-1}, and 0.001 mg·L^{-1}, indicating 0% mortality for these concentrations. Zebrafish in these tanks exhibited normal swimming behaviors comparable to those in the negative control tank, and the feeding regimen continued without any issues. However, at the concentration of 1.0 mg·L^{-1}, a 100% mortality rate

was observed, with all five fish found dead. This result indicates that adult zebrafish could not tolerate the 1.0 mg·L^{-1} concentration.

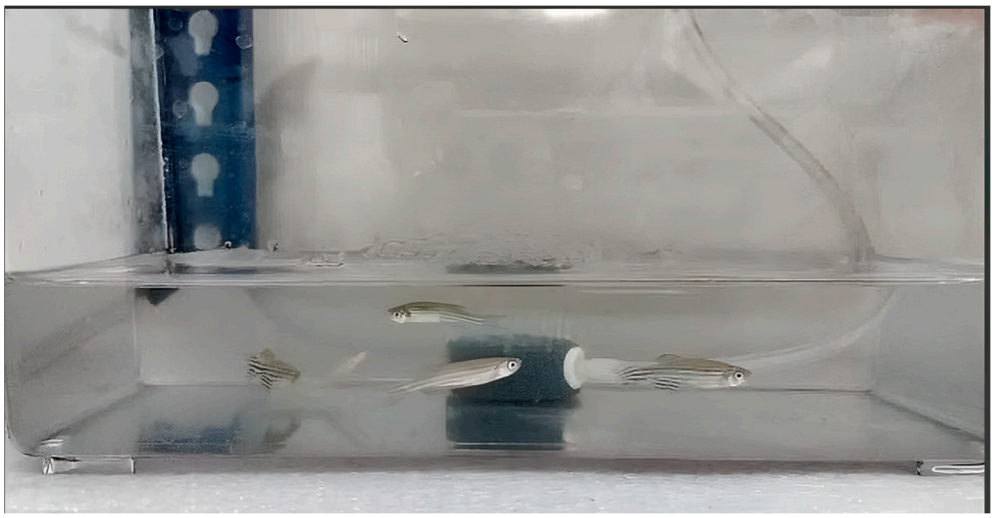

Figure 6. Zebrafish exposed to a 0.1 mg·L^{-1} concentration of NE-FLO™, displaying normal swimming patterns and responsive behavior towards external stimuli.

On the second day (48 h), observations remained consistent with those of day one. Concentrations of 0.1 mg·L^{-1}, 0.01 mg·L^{-1}, and 0.001 mg·L^{-1} continued to show 0% mortality, with zebrafish exhibiting normal living and swimming behaviors. By the third day (72 h), the final observations were similar to those of the previous days. Concentrations of 0.1 mg·L^{-1}, 0.01 mg·L^{-1}, and 0.001 mg·L^{-1} showed 0% mortality, and the fish remained alive and in good health, with no negative changes in swimming patterns, appetite, or excretions. Throughout the three days, the negative control tank showed no fish deaths, confirming that the water conditions did not contribute to mortality.

The estimated LC_{50} value for the tested emulsion is 0.316 mg·L^{-1}, indicating a high level of toxicity to adult zebrafish. According to standard toxicity classifications, substances with an LC_{50} value of less than 1 mg·L^{-1} are considered highly toxic. Despite this classification, the substance exhibited no toxicity at lower concentrations, with mortality occurring only at higher concentrations. This pattern suggests that the substance can be used safely at lower concentrations without posing significant risks to aquatic environments. However, at higher concentrations, the substance can cause significant mortality, necessitating stringent measures to manage and mitigate potential environmental impacts, especially in habitats supporting sensitive aquatic species. Further studies are recommended to explore the mechanisms of toxicity at higher concentrations and to assess the long-term ecological effects of the substance. Table 3 and Figure 7 illustrate a detailed overview of the acute toxicity test duration, results, log concentration (mg·mL^{-1}), and viability percentage.

Gu et al. [27] and Achenbach et al. [28] suggest investigating how toxicity operates at higher concentrations. Understanding whether the sample induces mortality by damaging specific organs, disrupting metabolic processes, or triggering other physiological stress responses can aid in developing better strategies to mitigate risks and establish safer application methods. Detailed studies, such as those on the heart-related effects of doxorubicin in zebrafish, illustrate how specific effects can be uncovered and potentially addressed [29].

Table 3. Results of the acute toxicity test on adult zebrafish.

	Sample Conc. ($mg.mL^{-1}$)	Log Conc. ($mg.mL^{1}$)	Day One (24 h)				Day Two (48 h)				Day Three (72 h)			
			No. of Fish (Initial)	No. of Fish (Final)	Total Deaths	Mortality %	No. of Fish (Initial)	No. of Fish (Final)	Total Deaths	Mortality %	No. of Fish (Initial)	No. of Fish (Final)	Total Deaths	Mortality %
Sample *	0.001	−2.3	5	5	0	0	5	5	0	0	5	5	0	0
	0.01	−1.5	5	5	0	0	5	5	0	0	5	5	0	0
	0.1	−0.8	5	5	0	0	5	5	0	0	5	5	0	0
	1	0.0	5	0	5	100								
Negative Control **	Filtered tap water		5	5	0									
	Average total deaths				0									

* Sample: Diluted NE-FLO™ treatment added to the brine shrimp with concentrations 0.001 $mg{\cdot}L^{-1}$, 0.01 $mg{\cdot}L^{-1}$, 0.1 $mg{\cdot}L^{-1}$, and 1 $mg{\cdot}L^{-1}$. ** Negative control: Filtered and conditioned tap water was used as a medium for the zebrafish.

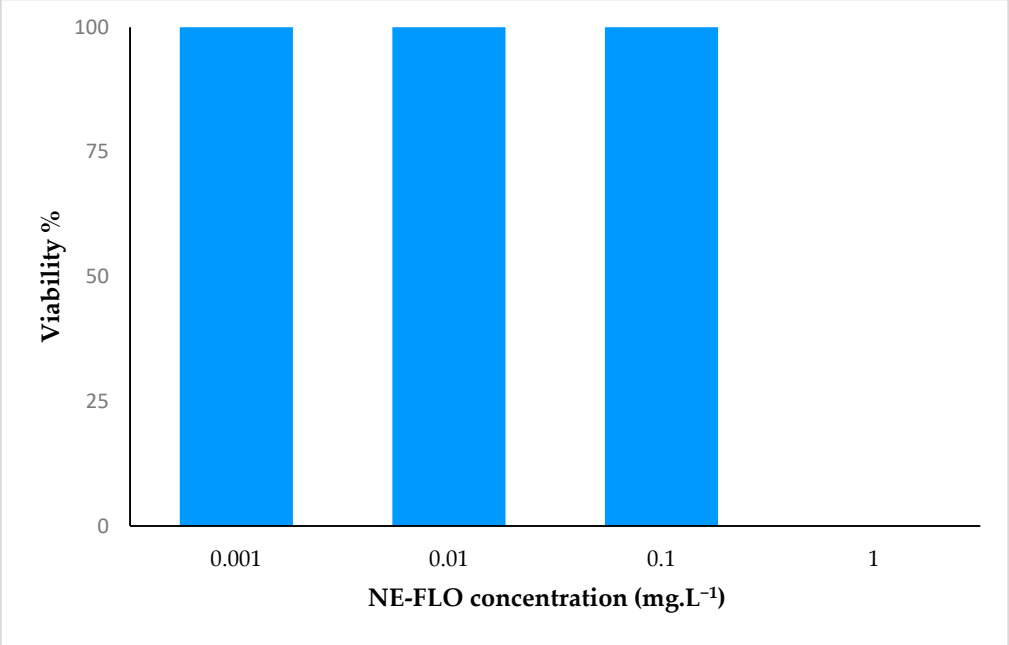

Figure 7. Relation between adult zebrafish viability percentage and NE−FLO™ concentration.

Zain et al. [30] reported on the use of flavonoid-enriched oil palm (*Elaeis guineensis* Jacq.) leaf extract (OPL) using the zebrafish model. Their study aimed to develop a flavonoid-enriched extract of OPL (OPL-FEE) and incorporate it into a nanoemulsion system (OPL-FEE-NE) to assess its toxicity, wound healing properties, and transcriptional effects using the zebrafish model. Based on embryonic toxicity assay results, they established test concentrations for the exposure experiments. Zebrafish mortality rates were monitored at 24, 48, 72, and 96 h, revealing a dose-dependent effect for the test samples, consistent with previous studies [31]. OPL-FEE exhibited high toxicity to adult zebrafish at 100 mg·L^{-1}, while OPL-FEE-NE showed toxicity at concentrations above 2 g·L^{-1}, resulting in 100% mortality within 24 h of exposure. However, after 96 h of exposure, no mortality was observed at test concentrations of 25 mg·L^{-1} for OPL-FEE and 0.5 g·L^{-1} for OPL-FEE-NE, indicating the safety of these concentrations for subsequent wound healing experiments.

4. Conclusions

The acute toxicity assessments conducted on brine shrimp and adult zebrafish followed appropriate protocols to ensure safety across a range of NE-FLO™ concentrations. These tests revealed a favorable safety profile, with minor mortalities observed in the brine shrimp toxicity test for 0.001 mg·L^{-1}, 0.005 mg·L^{-1}, and 0.01 mg·L^{-1} concentrations with increased mortality rates for higher concentrations of 0.1 mg·L^{-1}, 1.0 mg·L^{-1}, and 10 mg·L^{-1} in which the size of the organisms is taken into consideration. Similarly, no mortalities or adverse effects were observed in the acute toxicity evaluation on adult zebrafish at concentrations of 0·1 mg·L^{-1}, 0.01 mg·L^{-1}, and 0.001 mg·L^{-1}.

Additionally, the LC_{50} values for the nanoemulsion were 8.7474 mg·L^{-1} for brine shrimp and 0.316 mg·L^{-1} for adult zebrafish. They exhibited no toxicity at lower concentrations but caused mortality at higher ones, suggesting safe usage at lower doses and potential risks at higher doses, especially in sensitive aquatic environments.

These findings affirm the overall safety of fish oil nanoemulsions in acute toxicity tests, indicating potential for diverse industrial applications. This study lays a solid groundwork for further exploring nanoemulsions as safe and effective carriers for bioactive compounds. Nevertheless, continued studies are necessary to fully evaluate their safety and efficacy across broader applications and prolonged exposure durations.

5. Patents

This work was generated from the patent application of the formulation NE-FLO™, which is currently in the First Substantive Examination Adverse Report, Malaysian Patent Application PI 2022004802.

Author Contributions: A.A.H. performed the experiment, analyzed the data, validated the result, and wrote the manuscript. N.A.N.A. performed the nanoemulsion experiments. A.A.M.E. and N.A.-S. designed the research, obtained the funds, and co-supervised the first author. N.A.-S., H.M.S. and A.A.M.E., provided research resources, analyzed the data, and reviewed the manuscript. N.M.N. contributed to analyzing the data and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of IIUM Animal Care and Use Committee (I-ACUC), Research Management Center (RMC), Kuantan Campus, and approved by the Ethical Committee.

Data Availability Statement: Some data presented in this study are available upon request from the corresponding author due to patent processing.

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