Toxicity Test of Nanoemulsions of Nutmeg Fruits and Leaves Essential Oil against *Artemia salina* Leach and Its Cytotoxicity Test against Breast Cancer Cells T47D

Undri Rastuti[1*], Hartiwi Diastuti[1], Senny Widyaningsih[1], Moch Chasani[1], Cindi Sheiliyani[1], Anisa Rahmasari[1], Bunga Sita Roihanul Fajriyah[1], Puspa Rahma Mesayu[1], and Ranti Kamila Habibie[2]

[1]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Jenderal Soedirman, Jl. Dr. Soeparno No. 61, Karangwangkal, Purwokerto 53123, Indonesia*

[2]*Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia*

* ***Corresponding author:***

email: undri.rastuti@unsoed.ac.id

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Abstract: *Nutmeg (*Myristica fragrans *Houtt) is a widely known spice plant, which has been reported to offer several benefits. Therefore, this study aims to develop and analyze nanoemulsions of nutmeg leaves and fruit essential oil, as well as determine their toxicity and cytotoxicity. Nanoemulsions were formulated with varying concentrations of essential oil, including 0, 1, 2, 4, and 6%. Characterization included organoleptic assessment, pH measurement, type examination, viscosity testing, transmittance analysis, particle size distribution measurement, centrifugation, and freeze-thaw cycle test. Toxicity testing results using the brine shrimp lethality test (BSLT) showed that nanoemulsions were toxic except NF F4 with high toxicity. Cytotoxicity testing on T47D breast cancer cells showed moderate activity for NF F4 nanoemulsions (IC_{50}: 34.363 ppm), while NL nanoemulsions were deemed inactive (IC_{50}: 33576.430 ppm). In addition, the organoleptic characteristics of all nanoemulsions were stable, and most parameters met the desired standards. Based on the results, further studies exploring nanoemulsions with natural products must be carried out to determine their advantages, specifically in the development of sciences.*

Keywords: *nanoemulsion; essential oil of nutmeg fruits and leaves; toxicity; breast cancer cells T47D;* Artemia salina *Leach*

■ INTRODUCTION

Cancer is widely known to be among the leading global causes of mortality. In addition, it comprises a set of medical conditions distinguished by the irregular proliferation of cells and their unregulated growth within the body. Several studies have shown that this condition can ultimately lead to fatality without effective management of its proliferation. Based on a report by the World Health Organization (WHO), cancer patients constitute approximately 0.14% of the Indonesian population. The number of cancer-related deaths in Indonesia has also been reported to reach 22,000 from a total of 396,914 new cases [1]. The most common type of cancer suffered by humans is breast cancer, accounting for approximately 11.7% of all cases [2].

According to previous studies, current breast cancer therapy has several limitations, such as adverse side effects, low efficacy, and high therapeutic costs [2]. Common medications, including bevacizumab, cyclophosphamide, cisplatin, methotrexate, and paclitaxel, are known to carry a range of significant side effects, such as thromboembolic events, gastrointestinal perforation, pulmonary embolism, cerebral hemorrhage, gastrointestinal hemorrhage, hypersensitivity reactions, anaphylaxis, and hair loss. This shows a pressing need to develop alternative treatment options with minimal adverse effects.

Over the years, plants have served as a fundamental source for creating medicinal remedies aimed at preventing and treating various diseases. A significant surge in studies and development efforts focused on

natural products, with a particular emphasis on their potential incorporation into clinical applications, including cancer treatment, has also been observed [3]. An Indonesian plant with anticancer activity is nutmeg (*Myristica fragrans* Houtt), which is a spice possessing several benefits. Previous reports have shown that nutmeg can be used in pharmacological fields as an anticancer, anti-inflammatory, antimicrobial, antioxidant, and antidepressant agent [4].

In line with previous reports, nutmeg is a significant bioreactor, yielding essential oil as its main product, which has high economic value [5]. Rastuti et al. [6] reported that secondary metabolites in the essential oil of nutmeg leaves and fruits contain β-pinene, γ-terpinene, sabinene, α-pinene, 4-terpineol, limonene, and myristicin. This content has the potential to be further developed as a medicinal ingredient. A typical example is myristicin, which can inhibit cancer cell growth, serving as an active anticancer compound [4].

Essential oil is remarkably sensitive and prone to degradation when exposed to external factors, such as oxidation, evaporation, heat, and light. In addition, their low solubility in water and high volatility limit the direct use of the components without a pharmaceutical carrier. Encapsulation methods are often used to address the limitations, often comprising vesicular or particulate delivery systems. One such approach comprises the use of micro or nanoemulsions [7]. Several reports showed that nanoemulsions were transparent emulsion systems that were a combination of oil and water stabilized by surfactant molecules with a droplet size ranging from 5–200 nm [8].

Nanotechnology can reduce particles to nano size (10^{-9} m), which has the potential to increase the efficiency and effectiveness of essential oil ingredients, as well as minimize side effects and toxic reactions [9]. These nanosystems in vesicular form have been shown to enhance the bioavailability and diffusion of essential oil due to their diminutive droplet size while also contributing to their properties through the wetting capability of surfactants [7]. The small particle size causes nanoemulsions to be kinetically stable and thermodynamically, leading to their use as drug delivery systems [10]. Therefore, this study aims to determine the toxicity and cytotoxicity of nutmeg leaves (NL) and nutmeg fruits (NF) essential oils, and their nanoemulsions. To assess toxicity, the brine shrimp lethality test (BSLT) method was used on the larvae of *Artemia salina* Leach shrimp, while cytotoxic was performed against T47D breast cancer cells.

■ EXPERIMENTAL SECTION

Materials

The ingredients used in this study were nutmeg essential oil obtained from the refining industry in Dayeuhluhur, Cilacap, Indonesia; Tween surfactant 80 p.a., propylene glycol p.a., methylene blue, dimethyl sulfoxide (DMSO) from Merck, Germany, *A. salina* eggs, seawater, yeast (fermipan), and aquadest.

Instrumentation

The tools used in this study were test glassware, fillers, micropipettes, digital pH meters (Ohaus starter 5000), ovens, Ostwald viscometer, hot plates, pycnometers, magnetic stirrers, UV-vis spectrophotometer (Shimadzu 1800), a set of *A. salina* Leach egg hatching devices, incandescent lamps, vial bottles, aerators, and particle size analyzer (PSA) Microtac Flex 11.1.0.6.

Procedure

Nanoemulsions formulation

Nanoemulsion formulation was prepared with variations of oil, propylene glycol, and Tween 80, as presented in Table 1, thereby forming the oil phase. Each variation was homogenized using a magnetic stirrer at a speed of 750 rpm and a temperature of 50 °C for 60 min. Subsequently, it was slowly supplemented with aquadest up to a volume of 100 mL. Re-homogenization was carried out using a magnetic stirrer for 540 min at a temperature of 50 °C and speed of 1,250 rpm.

Characterization of nanoemulsions

An organoleptic test was performed to identify the characteristics of nanoemulsions by observing changes in color, odor, phase separation, and clarity. pH measurement was carried out using a pH meter by the

Table 1. Nanoemulsions formula of nutmeg leaves essential oil (NL) and nutmeg fruits essential oil (NF)

Formula		Essential oil (v/v)	Tween 80 (v/v)	Propylene glycol (v/v)	Distilled water (v/v)
F0	NL	0	20	15	Scaled up to 100
	NF	0	20	15	
F1	NL	1	20	15	
	NF	1	20	15	
F2	NL	2	20	15	
	NF	2	20	15	
F3	NL	4	20	15	
	NF	4	20	15	
F4	NL	6	20	15	
	NF	6	20	15	

device and placed into a container containing nanoemulsions until the pH value on the meter was read. Nanoemulsions type examination was carried out using the dye test method, which was performed by dripping dyes into nanoemulsions. In addition, nanoemulsions gave color to the dispersed area. When nanoemulsions showed a non-uniform color, it was considered to be water in oil (w/o), and the uniform color showed nanoemulsions were oil in water (o/w).

Viscosity measurement was carried out using an Ostwald viscometer by inserting 10 mL of nanoemulsions preparation into the device. The liquid in the Ostwald viscometer was sucked with filler until it crossed the upper limit. In addition, the time of the decline of the preparation from the upper limit mark to the lower limit mark of the Ostwald viscometer was recorded and the viscosity (cP) was calculated. Percent transmittance test was performed using UV-vis spectrophotometry with a wavelength of 650 nm. Particle size distribution was measured using PSA Microtac Flex 11.1.0.6 and conducted at the Physics Laboratory, Yogyakarta State University, Yogyakarta, Indonesia. Kinetic stability testing was carried out by inserting 2 mL nanoemulsions preparations into an Eppendorf tube. Subsequently, the tube was centrifuged at a speed of 6,000 rpm for 30 min, followed by observation of physical characterization. Thermodynamic stability testing was performed using the freeze-thaw cycle method by storing the preparation at a temperature of 4 °C for 24 h, then transferring it at 40 °C for 24 h (1 cycle). The test was carried out in a total of 3 cycles.

Toxicity and cytotoxicity test with BSLT method

Hatching larvae of *A. salina* Leach. A set of hatchery devices and aerators as a source of oxygen was prepared and 1 L of seawater was placed into the hatchery box. The hatchery box was divided into 2 parts, where the dark part was covered with black cloth and the light part was illuminated using an incandescent lamp. A total of 0.1 g of *A. salina* L eggs were soaked in aqueous and allowed to stand for a while. The floating eggs were discarded while the drowned eggs were taken and hatched in hatching boxes on the dark part by adding yeast solution as a nutrient. Hatching of eggs was carried out for 48 h, with the hatched *A. salina* L eggs being moved toward the bright part of the hatching box.

Manufacture of NL and NF nanoemulsions test solutions. A total of 0.05 g of NL and NF nanoemulsions preparations were each taken, dissolved in 1.00 mL DMSO, and added to seawater until the volume became 100 mL (500 ppm).

Toxicity test with BSLT method. A total of 10 *A. salina* L were taken from the drip box and placed in the test tube. The test solution was added and diluted with 1% DMSO to a volume of 5 mL so that the final concentration was 0, 5, 10, 50, 100, and 150 ppm. The test tube was incubated in an open state and received light for a total of 24 h. The number of surviving shrimps was calculated, and the percentage of shrimp mortality

was obtained to determine the LC_{50} value. Each concentration was carried out in 3 repetitions (*triplo*).

Cytotoxicity test against T47D cells. The NL and NF nanoemulsions formulas with the smallest LC_{50} values were tested *in vitro* against T47D breast cancer cells using the MTT assay method. This test was conducted at *Puspitek Riset Teknologi Pengujian dan Standar* – BRIN, Serpong, Tangerang City, Banten, Indonesia.

Data analysis

The toxicity of NL and NF nanoemulsions formulas could be determined by looking at the magnitude of the value of LC_{50}, which killed *A. salina* L up to 50%. In addition, analysis was carried out using the SPSS Statistics 17.0 for Windows program with probit regression analysis.

■ RESULTS AND DISCUSSION

Nanoemulsions Formulation

The constituent components of nanoemulsions were the essential oil of NL and NF, Tween 80, propylene glycol, and water. Tween 80 surfactant helped to reduce surface tension, whereas the large surfactant composition used could make the size of the resulting nanoemulsion smaller. Propylene glycol cosurfactant played an essential role in increasing the solubilization of nonpolar groups and helped surfactants work to lower the interfacial voltage of the water and oil phases. This was because the cosurfactant molecule was positioned between surfactants. A mixture of surfactants and cosurfactants with essential oils of NL and NF could produce stable nanoemulsions preparations [11]. NF and NL nanoemulsions were made by spontaneous emulsification with a magnetic stirrer to produce clear and transparent nanoemulsions, as presented in Fig. 1. Stirring was not performed too fast or slow because it had the potential of becoming foamy and not homogeneous [12].

Characterization of Nanoemulsions

Organoleptic test

The organoleptic assay helped to describe the physical state of NF and NL nanoemulsions. The results of the NF nanoemulsions test and NL color parameters showed that the greater the concentration of nanoemulsions preparations, the more yellow the color produced, as presented in Fig. 2. This was because of the increasing use of nutmeg essential oil in preparations that had a characteristic yellow color. The results of the organoleptic test of aroma parameters in Fig. 3 showed that the higher the concentration, the greater the intensity of the aroma produced. This was because the essential oil

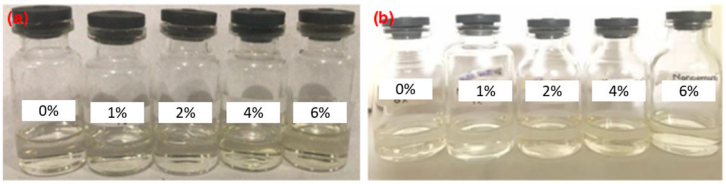

Fig 1. Nanoemulsion results of (a) NF (b) NL

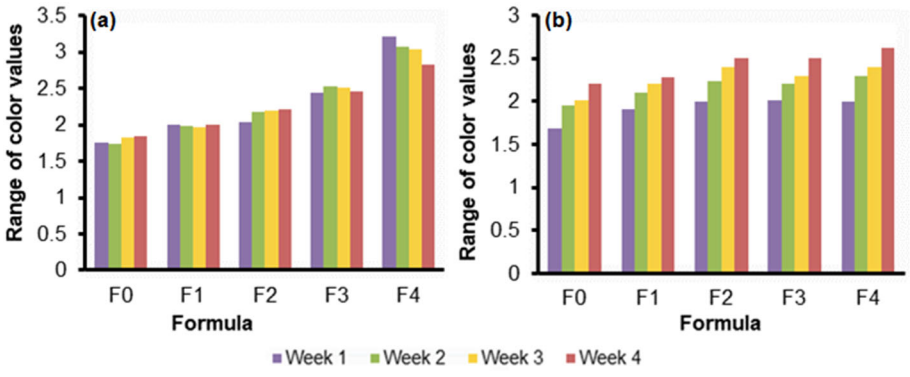

Fig 2. Color parameter organoleptic test graph of (a) NF and (b) NL

of NF and NL had a distinctive aroma property. The reduced aroma of essential oil nanoemulsions from weeks 1 to 4 occurred due to an oxidation reaction between oxygen in the open-air binding to unsaturated fatty acids in nutmeg essential oil to form peroxide compounds [13].

Based on the results of the clarity parameter organoleptic test in Fig. 4, all nanoemulsions preparations were clear. In addition, the decrease in the clarity of nanoemulsion preparations at week 4 was caused by a decrease in the effectiveness of surfactants to ensure that nanoemulsion droplets united to produce a larger droplet size [14]. The result of the last organoleptic test was the phase separation parameter, as presented in Fig. 5. All NF and NL nanoemulsion preparations did not undergo phase separation to enhance their stability against physical strength.

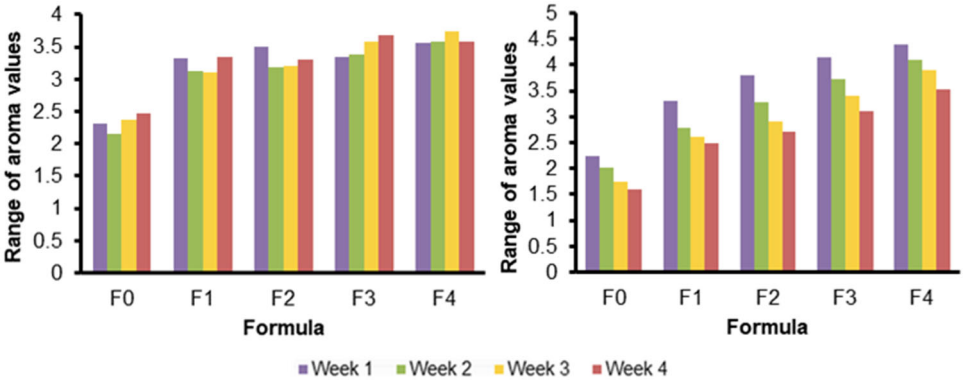

Fig 3. Organoleptic test graph of aroma parameters of (a) NF and (b) NL

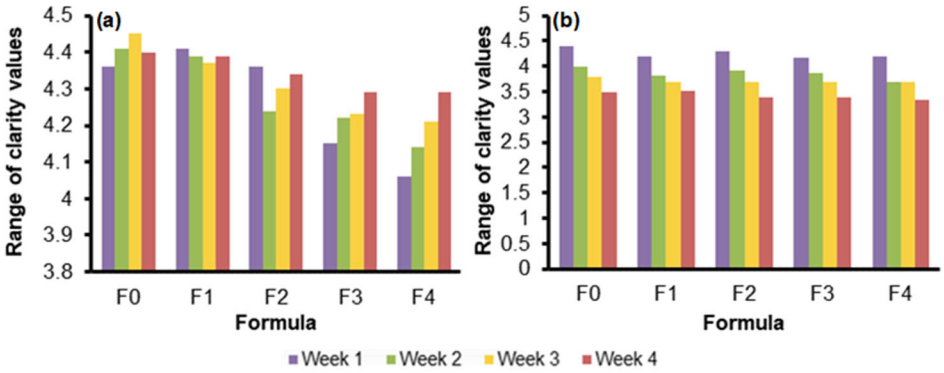

Fig 4. Organoleptic test graph of clarity parameters of (a) NF and (b) NL

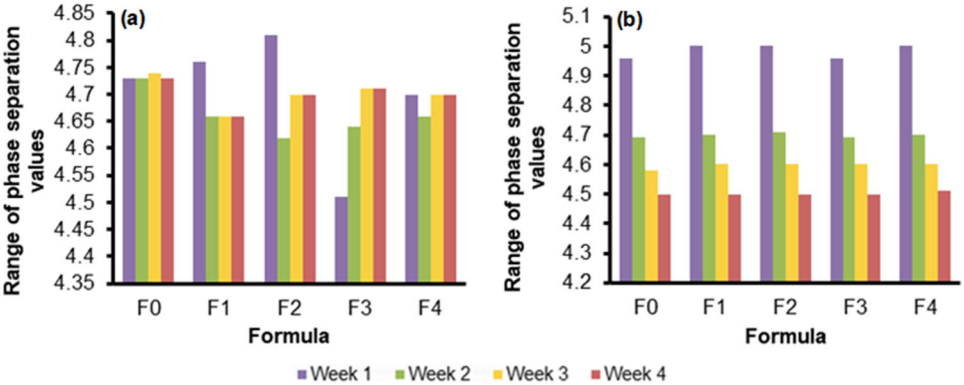

Fig 5. Organoleptic test graph of phase separation parameters of (a) NF and (b) NL

pH measurement

The pH value results in Fig. 6 showed that NL nanoemulsions were lower than NF in each formula. However, all pH values of the resulting nanoemulsion preparations were safe to use as basic ingredients for drugs because their pH was in line with the small intestine (5–7) [15], the main organ of drug absorption.

Nanoemulsions type inspection

The examination was carried out by dripping methylene blue on NL and NF nanoemulsions preparations. Based on Fig. 7, NL and NF nanoemulsions preparations could dissolve methylene blue. This showed that the preparation was o/w nanoemulsions type. The o/w nanoemulsions type occurred because the oil phase had been dispersed in the water phase [15].

Viscosity measurement

Viscosity measurements were carried out to determine the viscosity of nanoemulsions preparations. Fig. 8 showed that NL and NF nanoemulsions preparations had good viscosity and were in the nanoemulsions viscosity range, namely 10–2000 cP [16]. The viscosity of nanoemulsion preparations affected the release of active substances from plants [17].

Transmittance percent test

All NL and NF nanoemulsions preparations had a high transmittance percent value; hence, the resulting nanoemulsion preparations were clear and transparent, as presented in Fig. 9. In addition, good nanoemulsions had a transmittance close to 100%, which was similar to water [12].

Particle size distribution measurement

The principle of measuring particle size distribution using PSA was the presence of dynamic light scattering (DLS) to measure the size distribution of particles experiencing Brownian motion, which were then

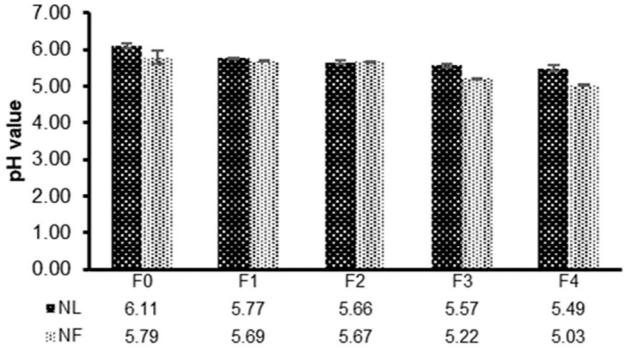

Fig 6. Graph of nanoemulsions pH measurement results

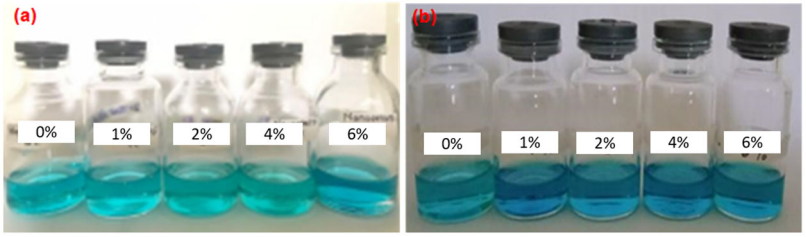

Fig 7. Nanoemulsions type examination results of (a) NL and (b) NF nanoemulsions

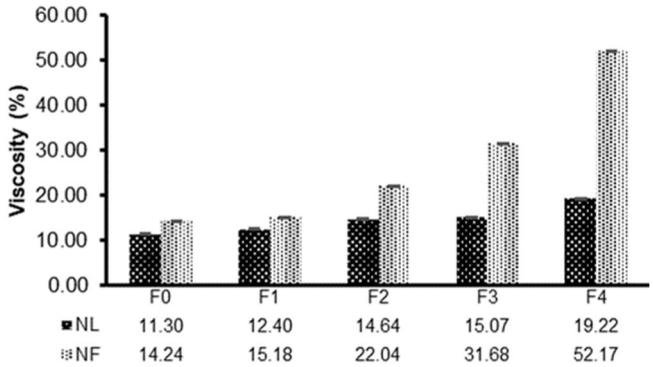

Fig 8. Graph of nanoemulsions viscosity measurement results

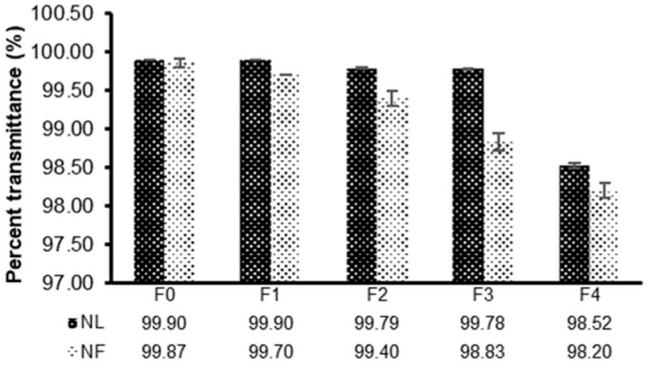

Fig 9. Graph of percent transmittance test result of nanoemulsions

Table 2. Particle size distribution results of nanoemulsions

Formula		Particle size (nm)	Volume (%)	PDI
F1	NL	13.870	89.400	0.090
		134.500	10.600	
	NF	13.890	82.200	0.427
		93.500	17.800	
F2	NL	15.070	84.400	0.221
		114.200	15.600	
	NF	16.460	74.500	1.625
		172.200	25.500	
F3	NL	15.480	76.400	1.423
		164.600	23.600	
	NF	19.370	71.600	1.374
		188.500	28.400	
F4	NL	16.600	70.400	0.428
		179.100	29.600	
	NF	19.570	70.200	1.235
		256.800	29.800	

read by the photon detector at a certain angle so that the particle size could be determined [18]. This method was considered more accurate than image analysis techniques, such as SEM and TEM, because it used laser light as an information medium for measuring objects (particles). In addition, the measurement time was faster because light had a very large propagation speed and could transmit information in a very short time [19].

Based on Table 2, almost all NL and NF nanoemulsion preparations had nanoparticle droplet sizes except NF F4 nanoemulsions. The polydispersity index (PDI) showed the homogeneity of the particle size. A PDI value of < 0.5 showed a uniform particle size but had a distinct shape and wide particle distribution. A PHI value greater than 0.5 showed that the globule size was not comparable, the shape was irregular, and the particle dispersion was disorganized [12]. As presented in Table 2, NF data from F2, F3, and F4 nanoemulsions and NL F3 had PDI values of more than 0.5, showing that the samples had a very broad particle size distribution. Stirring too quickly caused nanoemulsion particles to collide thereby becoming large and turbid particles, while slow stirring could cause inhomogeneity [18]. In addition, the insufficiency of Tween 80 surfactant molecules used in the manufacture of nanoemulsions preparations could also cause coalescence [12].

Fig. 10 and 11 showed graphs of the distribution particle size of nanoemulsions, where almost all samples had 2 peaks. A total of 2 peaks were consistently identified by the DLS measurements, suggesting that the sample was characterized by a bimodal distribution of particle size. The first peak, having the larger number of particles, was centered around 34.8 ± 8.4 nm and was associated with a higher frequency/probability of result particles within the size range. The second peak was spread over a larger range, namely 778 to 5070 nm, and the frequency of resulting larger particles was smaller [20].

Kinetic stability test

The kinetic stability test was performed using centrifugation, and the results for the kinetic stability test

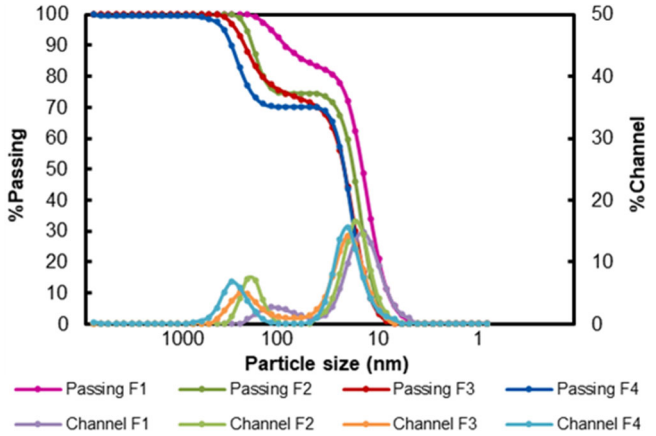

Fig 10. Particle size distribution graph of NF essential oil nanoemulsions

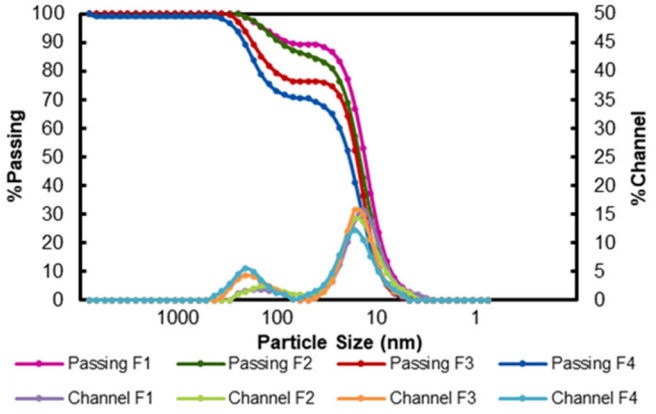

Fig 11. Particle size distribution graph of NL essential oil nanoemulsions

are presented in Table 3. All resulting NL and NF nanoemulsions were stable as there was no phase separation or precipitation, which remained clear. Centrifugation helped to determine whether there was a phase separation that occurred due to the force of gravity so that nanoemulsions could be said to be stable [12].

Freeze-thaw cycle test

The freeze-thaw cycle test aimed to assess the preparation. Nanoemulsions remained stable at varying temperatures at specific time intervals. Table 4 shows the results of the freeze-thaw cycle test for 3 cycles of nanoemulsions preparations. The results showed that there was no difference in terms of color, aroma, clarity, phase separation, and precipitation, hence, NL and NF nanoemulsions were stable against temperature changes.

Toxicity Test against *A. salina* Leach

A toxicity test using the BSLT method aimed to determine the toxic effect of NL and NF nanoemulsions on biological systems to ensure that the results obtained can be used for other bioactivity test images. The resulting *A. salina* L shrimp mortality was used to determine the lethal concentration value (LC_{50}). A sample was very toxic when it had an LC_{50} value of < 30 ppm, toxic when it was 30–1000 ppm, and non-toxic when it was > 1000 ppm [21]. Table 5 showed that NL F1, F2, F3, and F4 nanoemulsions and NF F1, F2, and F3 nanoemulsions were toxic while NF F4 nanoemulsions were highly toxic. NF nanoemulsions had a better toxicity value because the content of secondary metabolite compounds in the form of myristicin in NF was higher, namely 22.30–36.05% [5] while NL had 3.28–4.46% [22].

Cytotoxicity Test against T47D Cells

The cytotoxicity test aimed to determine the effect of anticancer molecules on a medicinal plant [23]. In this study, a cytotoxicity test was carried out on NF and NL nanoemulsions against breast cancer cells (T47D) using IC_{50} parameters. Based on the National Cancer Institute (NCI), a compound was said to have active anticancer activity when it had an IC_{50} < 30 ppm, an active rate when

Table 5. LC_{50} values of NL and NF nanoemulsions

Formula		LC_{50}
F1	NL	122.141
	NF	45.742
F2	NL	91.335
	NF	42.725
F3	NL	46.082
	NF	30.798
F4	NL	35.211
	NF	27.869

Table 3. Centrifugation test results of nanoemulsions

Observation after centrifugation	Nanoemulsions preparations									
	F0		F1		F2		F3		F4	
	NL	NF	NL	NF	NL	NF	NL	NF	NL	NF
Turbidity	N	N	N	N	N	N	N	N	N	N
Phase separation	N	N	N	N	N	N	N	N	N	N
Precipitation	N	N	N	N	N	N	N	N	N	N

Information: N = None

Table 4. Freeze-thaw cycle nanoemulsions test results

Observation	NL and NF nanoemulsions preparation									
	Before freeze-thaw cycle test					After freeze-thaw cycle test				
	F0	F1	F2	F3	F4	F0	F1	F2	F3	F4
Color	CS	CS	CS	CS	CS	CS	CS	CS	CS	CS
Clarity	C	C	C	C	C	C	C	C	C	C
Aroma	DA	DA	DA	DA	DA	DA	DA	DA	DA	DA
Phase separation	N	N	N	N	N	N	N	N	N	N

Information: CS = Colorless, C = Clear, DA = Distinctive aroma, N = None

Table 6. IC_{50} values of NL and NF nanoemulsions

Nanoemulsion Formula		IC_{50} (ppm)
F4	NF	34.363
	NL	33,576.430

it had a value of $30 \leq IC_{50} \leq 100$ ppm and inactive when it was > 100 ppm [24]. Table 6 showed that NF nanoemulsions were included in the moderately active category against T47D cells with an IC_{50} value of 34.363 ppm, while NL nanoemulsions were included in the inactive category with an IC_{50} value of 33,576.430 ppm.

■ CONCLUSION

NL and NF essential oil nanoemulsions had good organoleptic properties and physical stability with a dominant droplet size of less than 10 nm. The results of testing the toxicity activity of nanoemulsions against *A. salina* Leach showed that NL essential oil F1, F2, F3, and F4 nanoemulsions and NF essential oil F1, F2, and F3 nanoemulsions were toxic while NF essential oil F4 nanoemulsions were highly toxic. In addition, the toxicity activity of nanoemulsions against T47D cancer cells showed that NF essential oil was included in the active category compared to NL.

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■ CONFLICT OF INTEREST

The authors declare no conflicts of interest.

■ AUTHOR CONTRIBUTIONS

Undri Rastuti, Hartiwi Diastuti, Senny Widyaningsih, Moch Chasani, Cindi Sheiliyani, Anisa Rahmasari designed, enhanced the discussions, and conducted the experiments. Undri Rastuti, Bunga Sita Roihanul Fajriyah, Puspa Rahma Mesayu wrote the draft of the manuscript and conducted the experiment and calculations. Ranti Kamila Habibie participated in designing and revising the manuscripts. All authors agreed to the final version of this manuscript.

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