



Development of nanostructured lipid carrier containing tea tree oil: Physicochemical properties and stability

[Desarrollo de un portador lipídico nanoestructurado que contiene aceite del árbol del té: Propiedades fisicoquímicas y estabilidad]

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Abstract

Context: Tea tree oil (TTO) is an essential oil derived from *Melaleuca alternifolia*, with high antimicrobial and antifungal potential. Unfortunately, its topical antifungal efficacy is limited because it is volatile, thermolabile and easily oxidized. A formulation has been developed to overcome this problem by encapsulating TTO in a nanostructured lipid carrier (NLC).

Aims: To determine the effect of the liquid to solid lipid ratio on the physicochemical properties and the stability of TTO-loaded NLC.

Methods: Five formula of TTO-loaded NLCs were produced by high shear homogenization method and characterized according to their particle size, size distribution, polydispersity, zeta potential, thermal characteristics, X-ray diffraction, and terpinen-4-ol concentration. In addition, a stability study was conducted by observing its physical and chemical characteristics during storage in the refrigerator ($4 \pm 2^\circ\text{C}$) and at room temperature ($27 \pm 2^\circ\text{C}$) for six months.

Results: The resulting TTO-loaded NLC had an average droplet size under 400 nm. The particle size increases with increasing amount of liquid lipid in the formula. There were insignificant changes in organoleptic properties, polydispersity index, zeta potential and terpinene-4-ol concentration during stability study for six months. However, the particle size slightly increased during the six months of storage. Furthermore, the NLC 3, which formulated with a 25:95 ratio liquid to solid lipid, was chosen as the best formula, since it demonstrated the best physicochemical characteristic and stability.

Conclusions: TTO-loaded NLC with good physicochemical characteristics and stability has been successfully developed. In addition, NLC 3 is considered as the best NLC formula, which exhibits characteristics and stability that meet the requirements.

Keywords: differential scanning calorimetry; nanostructured lipid carrier; physicochemical stability; tea tree oil; terpinen-4-ol.

Resumen

Contexto: El aceite del árbol del té (TTO) es un aceite esencial derivado de *Melaleuca alternifolia*, con un alto potencial antimicrobiano y antifúngico. Desgraciadamente, su eficacia antifúngica tópica es limitada porque es volátil, termolábil y se oxida fácilmente. Se ha desarrollado una formulación para superar este problema encapsulando TTO en un portador lipídico nanoestructurado (NLC).

Objetivos: Determinar el efecto de la proporción entre lípidos líquidos y sólidos sobre las propiedades fisicoquímicas y la estabilidad del NLC cargado con TTO.

Métodos: Se produjeron cinco fórmulas de NLC cargadas con TTO mediante el método de homogeneización de alto cizallamiento y se caracterizaron según su tamaño de partícula, distribución de tamaño, polidispersidad, potencial zeta, características térmicas, difracción de rayos X y concentración de terpinen-4-ol. Además, se realizó un estudio de estabilidad observando sus características físicas y químicas durante su almacenamiento en el frigorífico ($4 \pm 2^\circ\text{C}$) y a temperatura ambiente ($27 \pm 2^\circ\text{C}$) durante seis meses.

Resultados: La NLC cargada con TTO resultante tenía un tamaño medio de gota inferior a 400 nm. El tamaño de partícula aumenta con el incremento de la cantidad de lípido líquido en la fórmula. Se produjeron cambios insignificantes en las propiedades organolépticas, el índice de polidispersidad, el potencial zeta y la concentración de terpineno-4-ol durante el estudio de estabilidad durante seis meses. Sin embargo, el tamaño de las partículas aumentó ligeramente durante los seis meses de almacenamiento. Además, la NLC 3, formulada con una proporción 25:95 de lípidos líquidos y sólidos, fue elegida como la mejor fórmula, ya que demostró las mejores características fisicoquímicas y de estabilidad.

Conclusiones: Se ha desarrollado con éxito un NLC cargado con TTO con buenas características fisicoquímicas y estabilidad. Además, la NLC 3 se considera la mejor fórmula de NLC, que presenta características y estabilidad que cumplen los requisitos.

Palabras Clave: aceite del árbol del té; calorimetría diferencial de barrido; estabilidad fisicoquímica; portador lipídico nanoestructurado; terpinen-4-ol.

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INTRODUCTION

Tea tree oil (TTO) is an essential oil steam-distilled from *Melaleuca alternifolia* leaves and branches that contains nearly hundreds of compounds, mainly monoterpenes and alcohols. Some of its major components are 1,8-cineole, α -terpinene, γ -terpinene, terpinen-4-ol and terpinolene (Payzar et al., 2013; Yadav et al., 2017). Such a combination of terpenes makes this versatile oil has potent anti-inflammatory, antimicrobial, and antifungal therapies (Payzar et al., 2013; Satchell et al., 2002; Sharifi-Rad et al., 2017; Yadav et al., 2017). The antifungal activity of tea tree oil has been extensively studied, and it has been found to be effective against a wide range of fungal species (Carson et al., 2006).

However, physicochemically, the components of TTO are volatile, thermolabile, and easily oxidized. The active substance concentration above the skin (as the site of absorption) is limited due to its volatile nature. Thermolabile substances can degrade when exposed to high temperatures, and a decline in the active substances quality and quantity will occur by result from oxidation. Due to these issues, TTO's pharmacological efficacy as a topical antifungal became was suboptimal. To address these problems, TTO must be encapsulated, e.g., in nanocarriers (Bunacurorso et al., 2021).

In recent years, nanocarrier drug delivery systems have been investigated as a means of enhancing the efficacy and safety of antifungal agents. Nanostructured lipid carriers (NLCs) are one such system that has been explored. NLCs are biocompatible and can improve the solubility and bioavailability of poorly soluble drugs, thereby enhancing their therapeutic potential. In the case of antifungal drugs, NLC formulations can increase drug delivery to the site of infection, reduce toxicity, and improve compliance (Nene et al., 2021).

NLC are the most prospective lipid-based nanocarrier system with high encapsulation and stable physicochemical. NLCs are formed by a matrix of solid lipid, liquid lipid, water, and stabilized with surfactants (Andrade et al., 2014; Pardeike et al., 2009). Many research has revealed the uses and benefits of NLCs as an essential oil carrier. Essential oils have numerous biological activities, but their use is limited due to their hydrophobic property, volatility and easily oxidized, making them susceptible to degradation when exposed to environmental stresses like oxygen, temperature, and light (Majeed et al, 2015). Encapsulation of essential oils in NLCs is a viable solution to overcome these limitations.

Radwan et al. (2022) reported that in comparison to the essential oils alone, the biological evaluation of NLC encapsulated fennel essential oil showed potential results on both larvicidal and adulticidal effects against *Culex pipiens*. Bunacurorso et al. (2021) developed NLC for intranasal administration of essential oils (*Lavandula*, *Mentha*, and *Rosmarinus*). The resulting NLC exhibits good stability, small particle size, homogeneous, and could be suggested as a potential add-on technique in the treatment of neurodegenerative disease when administered intranasally. According to Ghodrati et al. (2019), encapsulating peppermint essential oil (PEO) in NLCs was a good way to create topical formulations for wound healing since PEO-loaded NLC had a good encapsulation efficiency, narrow size distribution, and faster healing of an infected wound model. Carbone et al. (2018) also reported potential synergistic effect of the antioxidant ferulic acid and the anti-inflammatory *Lavandula* essential oil given in NLC in stimulating cell proliferation and migration, suggesting a novel approach to the treatment of wounds.

The ability of NLC to maintain the stability of essential oils has been reported in various studies. Ghodrati et al. (2019) developed NLC for the delivery of peppermint essential oil with excellent stability and antibacterial activity. Not only physical and chemical stability, but also increased antibacterial activity were also demonstrated by Fatemeh Nahr et al. (2018) who developed NLC for delivery of cardamom essential oil. The results of the accelerated stability test by Carbone et al. (2018) suggested that NLC was successful in maintaining the stability of various Mediterranean essential oils.

Encapsulating TTO in NLC is important for several reasons. First, NLC can protect the TTO from volatilization, oxidation, and degradation, which can decrease its effectiveness as an antifungal agent. Second, NLC can facilitate the targeted delivery of tea tree oil into the skin, which can increase its antifungal activity. Third, NLC can provide a sustained release of tea tree oil over time, which can prolong its therapeutic effect and reduce the frequency of administration (Li et al., 2016; Nene et al., 2021).

Based on our previous studies that screening 18 combinations of solid lipid and liquid lipids, the combination of stearic acid and Miglyol 812 has the smallest particle size and meets the requirements for topical delivery (Fitriani et al., 2022). Since this study was limited to only several characteristics of NLC TTO (particle size, polydispersity index and zeta potential), it is not possible to conclude whether the developed NLC was stable on long term storage. To overcome

this limitation, we develop a stability study to determine whether NLC TTO is physicochemically stable on long term storage.

This research aims to determine the effect of the liquid to solid lipid ratio on the physicochemical characteristics and stability of TTO-loaded NLC. All NLC formulations were physicochemically characterized to assess the effects of liquid-to-solid lipid ratio (Miglyol 812 and stearic acid) on the nanoparticle characteristics, particularly average particle size, polydispersity, zeta potential, thermal characteristics, wide-angle X-ray diffraction profiles, and physicochemical stability.

MATERIAL AND METHODS

Materials

The experiment used tea tree oil purchased from Biotek Prima Indoplus (Surabaya, Indonesia), Poloxamer 188 (Merck, Germany), and terpinen-4-ol (analytical grade, Sigma-Aldrich). Pharmaceutical-grade stearic acid, Miglyol® 812, and Span® 80 were purchased from commercial suppliers PT. Labtech Citra Persada (Surabaya, Indonesia). The experiment also used many other chemicals and solvents of analytical grade, which were purchased from retail suppliers.

Preparation of tea tree oil-loaded nanostructured lipid carriers

A high shear homogenization was used to prepare NLC dispersions from TTO. To create the oil phase, TTO was first dissolved in Miglyol® 812 then added to stearic acid and Span 80, which had been melted at approximately 70°C, with five different stearic acid and Miglyol® 812 weight ratios. Then, after being dissolved in distilled water, Poloxamer 188 was heated to 70°C, added drop by drop into the oil phase, and simultaneously homogenized at 25,000 rpm using a homogenizer (Ika T25 Ultra-Turrax, Staufen, Germany). The mixture was kept at 70°C for 15 min and let to cool to room temperature, at which point TTO-loaded NLCs were formed (Ghodrati et al., 2019). The formulas are shown in Table 1.

Characterization of tea tree oil-loaded nanostructured lipid carriers

Particle size, PDI, and zeta potential

The laser diffraction in this research used Delsa™ Nano Submicron Particle Size Analyzer to evaluate particle size distribution and polydispersity index (PDI), which measure the distribution of the nanoparticle population. A zetasizer (Nanotrak Wave Microtrac W3717, USA) was used to measure the zeta po-

tential, whose magnitude indicates the stability of the TTO-loaded NLC dispersions. The particle size analyzer was also used to determine electrophoretic mobility to measure the electric charge on the particle's surface. The prepared NLCs were then diluted in appropriate amount distilled water and put into a cuvette. Measurements were taken at 25°C room temperature, with three replicates (Aryani et al., 2021).

Thermal analysis

In this analysis, a DSC thermal analyzer (Mettler Toledo) was used for the differential scanning calorimetry (DSC). Four mg of samples were weighed in a tightly closed aluminum pan. Then, the analyzer was gradually heated to 100°C at a rate of 20°C/minute. The onset temperature, melting point and endothermic enthalpy of TTO loaded NLC were observed (Aryani et al., 2021).

X-ray diffraction (XRD)

This step determined the crystal phase of the TTO-loaded NLCs. These formulations were dried to create XRD samples. The radial component of the scattering intensity was recorded at an angle of 2-theta. XRD profiles of steric acid and TTO loaded NLC and the intensity of sharp peaks observed (Fatemeh-Nahr et al., 2018).

Assays

Terpinen-4-ol, a marker component of the TTO-loaded NLC, was characterized using the method proposed by Avonto et al. (2016) with modifications. NLC was diluted in 96% ethanol, then sonicated and filtered. Gas chromatography-mass spectrometry (Shimadzu GC-MS QP1020SE) was used in the terpinen-4-ol assay, with helium as the carrier gas at a constant pressure of 24 psi. For the TTO analysis, in the first 2 minutes, the temperature was set at 50°C before raised to 180°C at a heating rate of 20°C/min. The injector temperature was 250°C (separation ratio set to 25:1), and the injection was made in triplicate for each sample. The full scan mass spectrum was recorded at 70 eV electron energy from m/z 35 to 450.

Stability studies

In the physical stability test, the TTO-loaded NLCs were stored for six months at different temperatures: 4 ± 2°C and 27 ± 2°C. The sample's particle, zeta potential, and polydispersity index were analyzed (Makoni et al., 2019).

In the chemical stability test, the TTO-loaded NLCs were stored for six months at different temperatures: 4 ± 2°C and 27 ± 2°C. The terpinene-4-ol concentration was measured at 0, 2nd, 4th, and 6th month.

Table 1. Formulas of the tea tree oil-loaded nanostructured lipid carriers (NLC).

Ingredient	Composition in formula (%)				
	NLC 1	NLC 2	NLC 3	NLC 4	NLC 5
Tea tree oil	5.00	5.00	5.00	5.00	5.00
Stearic acid	14.21	12.63	11.05	9.47	7.89
Miglyol® 812	0.79	2.37	3.95	5.53	7.11
Poloxamer 188 and Span 80	5.00	5.00	5.00	5.00	5.00
Distilled water	75.00	75.00	75.00	75.00	75.00

Table 2. Particle size, polydispersity index (PDI), and zeta potential of the tea tree oil-loaded nanostructured lipid carriers (NLC) during six-month storage at 27 ± 2°C and 4 ± 2°C.

Particle size (nm)		PDI		Zeta potential (mV)		
Stored at 27°C						
Formula	Month 0	Month 6	Month 0	Month 6	Month 0	Month 6
NLC 1	237.0 ± 2.7*	291.2 ± 1.5**	0.2173 ± 0.1	0.1848 ± 0.0	-12.5 ± 0.1	-13.2 ± 0.3
NLC 2	245.4 ± 2.0*	289.0 ± 1.6**	0.2119 ± 0.2	0.2065 ± 0.2	-14.1 ± 0.2	-14.8 ± 0.8
NLC 3	297.0 ± 2.6*	296.5 ± 4.0	0.1477 ± 0.1	0.2310 ± 0.1	-14.4 ± 0.3	-13.3 ± 0.7
NLC 4	363.0 ± 3.6*	396.1 ± 3.6**	0.3455 ± 0.1	0.1954 ± 0.1	-14.7 ± 0.3	-12.0 ± 0.9
NLC 5	394.3 ± 3.2*	405.0 ± 3.0**	0.2757 ± 0.1	0.2494 ± 0.0	-13.1 ± 0.2	-13.8 ± 1.0
Stored at 4°C						
Formula	Month 0	Month 6	Month 0	Month 6	Month 0	Month 6
NLC 1	237.0 ± 2.7*	333.0 ± 5.2**	0.2173 ± 0.1	0.1436 ± 0.0	-12.5 ± 0.1	-16.1 ± 2.5
NLC 2	245.4 ± 2.0*	272.6 ± 12.5**	0.2119 ± 0.2	0.2462 ± 0.1	-14.1 ± 0.2	-14.4 ± 1.1
NLC 3	297.0 ± 2.6*	343.7 ± 39.5**	0.1477 ± 0.1	0.1408 ± 0.1	-14.4 ± 0.3	-14.2 ± 0.8
NLC 4	363.0 ± 3.6*	338.3 ± 27.5**	0.3455 ± 0.1	0.2666 ± 0.1	-14.7 ± 0.3	-14.4 ± 0.8
NLC 5	394.3 ± 3.2*	378.3 ± 18.9	0.2757 ± 0.1	0.3375 ± 0.1	-13.1 ± 0.2	-15.0 ± 1.9

*Data are reported as mean ± SD, n = 3; PDI: polydispersity index; *The particle size of NLC 1, NLC 2, NLC 3, NLC 4 and NLC 5 showed significant difference (p<0.05); **The particle size of NLC showed significant difference between month 0 and month 6 (p<0.05).

Statistical analysis

Particle size, PDI, zeta potential and assay data were statistically analyzed with the one-way analysis of variance (ANOVA) with a probability value (p<0.05). All data were reported as mean ± SD (n = 3). Thermal analysis and X-ray diffraction data were analyzed using descriptive statistics.

RESULTS

Physicochemical characteristics of tea tree oil-loaded nanostructured lipid carriers

Particle size, PDI, and zeta potential

Five formulas of TTO-loaded NLCs have been successfully developed with organoleptic semisolid to liquid, white, and opaque with a typical tea tree oil odor. All formulations had average particle sizes

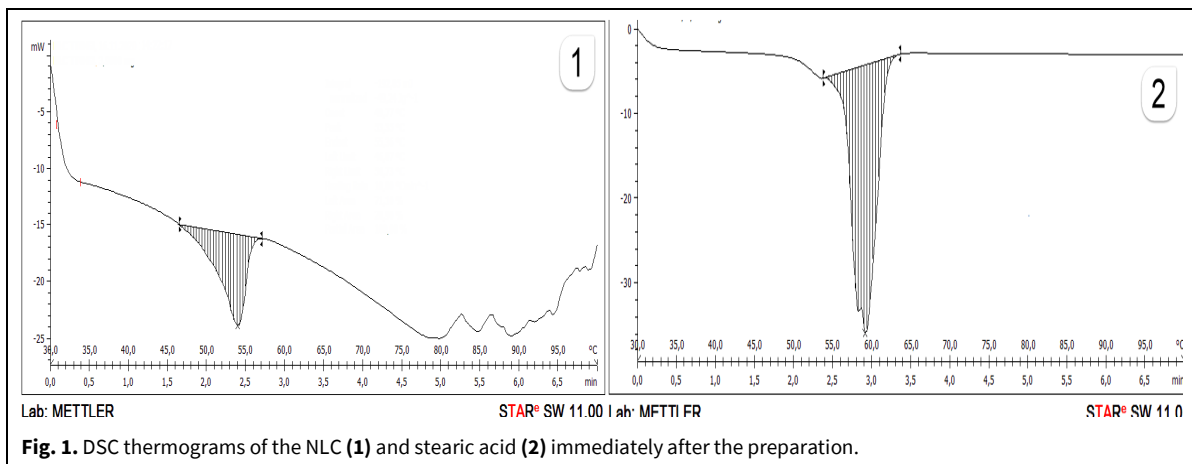
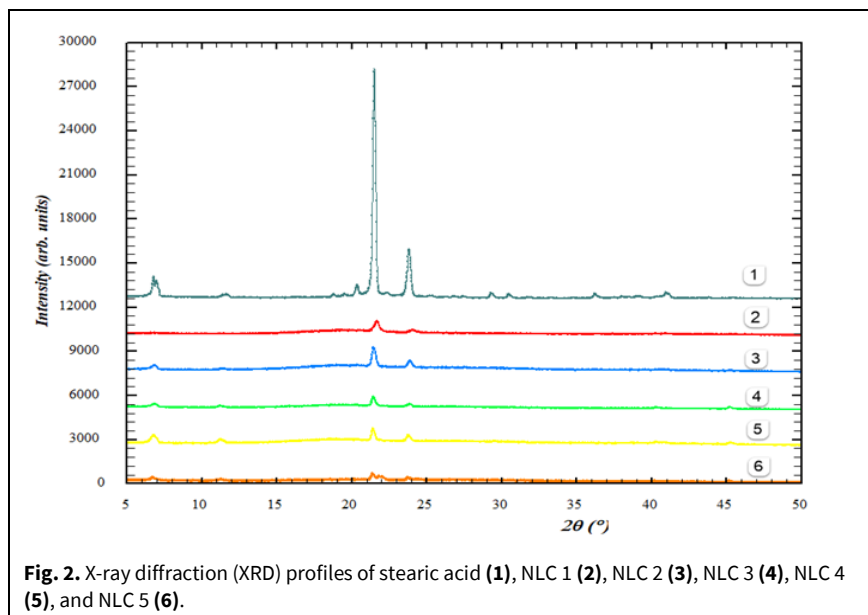
smaller than 400 nm, i.e., 237.4 nm (NLC 1) to 394 nm (NLC 5). NLC 1 and NLC 2 had the smallest particle sizes compared to other formulas. The polydispersity index (PDI) values for TTO loaded NLC formulation were between 0.147–0.345, indicating a relatively narrow size distribution. The zeta potential ranges between -12.5 to -14.7 mV for all the formulations. The results are shown in Table 2.

Thermal analysis results

The onset temperature, melting point and endothermic enthalpy of TTO loaded NLC are presented in Table 3. The onset temperature ranged from 49.77–46.30°C. NLC 1 exhibited the highest onset temperature while NLC 5 exhibit the lowest onset temperature. The endothermic enthalpy values for stearic acid and NLC 1 to 5 are -171.15 J/g, -49.24 J/g, -28.57 J/g, -16.54 J/g, -12.21 J/g and -3.58 J/g. NLC 1 shows the highest endothermic enthalpy value, while NLC5

Table 3. The differential scanning calorimetry (DSC) parameters: onset temperature, melting point, and enthalpy of stearic acid and tea tree oil-loaded nanostructured lipid carriers (NLC).

Formulation	DSC parameters		
	Onset (°C)	Melting point (°C)	Enthalpy (J/g)
Stearic acid	56.15	58.17	-171.15
NLC 1	49.77	53.35	-49.24
NLC 2	41.64	50.45	-28.57
NLC 3	45.51	49.00	-16.54
NLC 4	42.08	49.03	-12.21
NLC 5	46.30	46.96	-3.58

**Fig. 1.** DSC thermograms of the NLC (1) and stearic acid (2) immediately after the preparation.**Fig. 2.** X-ray diffraction (XRD) profiles of stearic acid (1), NLC 1 (2), NLC 2 (3), NLC 3 (4), NLC 4 (5), and NLC 5 (6).

shows the lowest endothermic enthalpy value. The DSC thermograms of the NLC 1 and stearic acid 2 immediately after the preparation are shown in Fig. 1.

X-ray diffraction (XRD) profiles

XRD profiles of steric acid and TIO loaded NLC has been shown in Fig. 2. The intensity of sharp peaks

at 21.3° belonging to NLC has decreased compared with stearic acid. Moreover, XRD peaks at 21.3° decreased from NLC 1 to NLC 5.

Terpinen-4-ol assay results

The terpinen-4-ol concentration on the NLC were measured and the result were presented in Fig. 3. The

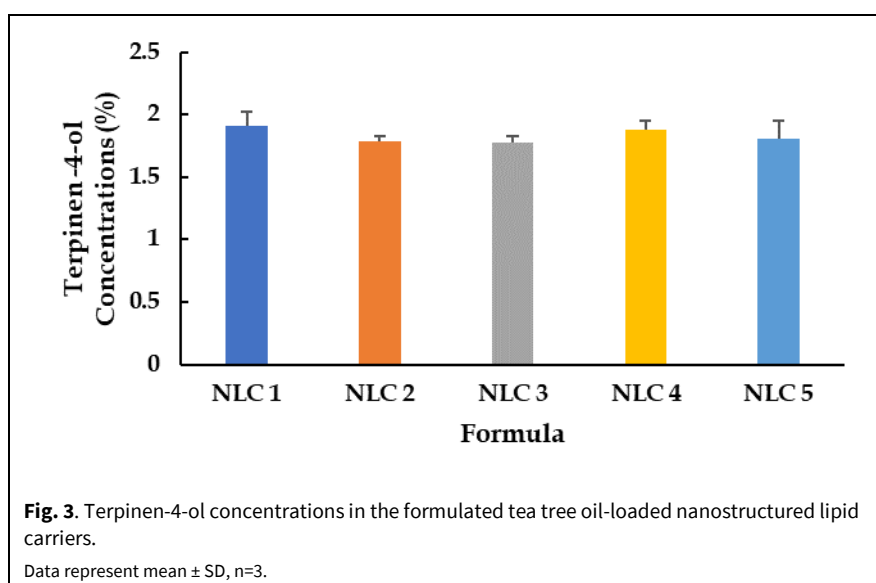
terpinene-4-ol concentration was 1.9079 % for NLC 1, 1.7845% for NLC 2, 1.7794% for NLC 3, 1.8794% for NLC 4 and 1.8135% for NLC 5. There was no significance difference in the terpinene-4-ol concentration in all NLC formulas.

Stability studies

The results of stability study during storage at 27°C and 4°C for 6 months revealed no significant changes in organoleptic properties, polydispersity index value and zeta potential of the TTO loaded NLC. At a storage temperature of 27°C, NLC TTO formula 3 showed a stable particle size for 6 months of storage. Meanwhile, the increase in particle size in NLC formulas 1, 2, 4 and 5 was detected at month 6.

The average particle size of NLC formula 1 changed from 237.07 nm to 291.23 nm. NLC formula 2 increased from 245.47.00 to 288.98 nm, NLC 4 increased from 363 to 396.1 nm and the average particle size of NLC formula 5 increased from 394.30 to 405 nm. At storage temperature of 4°C, all NLC formulas experienced a significant increase in particle size. The particle size (in mean), polydispersity index, and zeta potential during storage at 27 and 4°C are shown in Table 2.

Fig. 4. shows terpinen-4-ol concentrations in the nanostructured lipid carriers during six months of storage at 27°C and 4°C. There were no significant changes in the concentration of terpinen-4-ol when stored for six months.



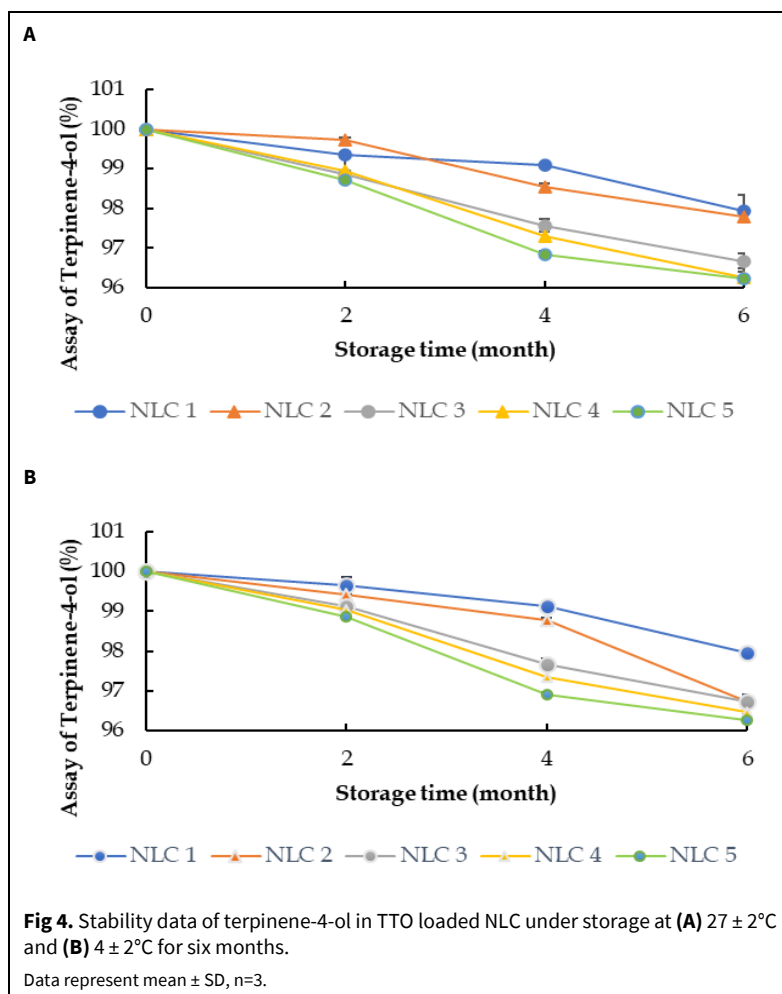
DISCUSSION

Particle size, PDI, and zeta potential

This research was designed to find out the effect of the liquid to solid lipid ratio on the physicochemical characteristics and stability of TTO-loaded NLC. In this study, five TTO-loaded NLC formulas were developed with a liquid to solid lipid ratio that increased from NLC 1 to NLC 5. The particle sizes produced in the five formulas ranged from 237.4 nm (NLC 1) to 394 nm (NLC 5). These results are in agreement with the particle size for NLC. The manufactured nanocarrier standard is usually between 50 and 400 nm (Mitrea and Meghea, 2014). This size range is associated with the high suitability of NLCs for a delivery system in topical fungal infection treatment (Nene et al., 2021). The small particle size represents good compatibility between the TTO (as the bioactive material), the solid lipid, and the liquid

lipid (Manzar et al., 2020). The size and structural properties of a lipidic nanocarrier are shaped by the type and concentration of its composition, e.g., lipids, emulsifiers, and bioactive compounds.

The results showed that there was an increase in particle size from NLC 1 to NLC 5. NLC 1 had the smallest particle size, and NLC 5 had the largest particle size. An increase in particle size was associated with an increase in the liquid-to-solid lipid ratio (Table 2). Ortiz et al. (2021) reported that, increasing the oil content from 5% to 20% led to an increase in particle size. The increase in particle size with increasing liquid lipid content could be attributed to the development of the nanoparticle core to accommodate the higher amount of Miglyol 812 in the formulation. In addition, the formation of a more irregular crystal structure occurred in the NLC system at the higher Miglyol 812 content, which in turn resulted in larger NLC particles.



The level of particle heterogeneity is displayed by polydispersity index (PDI). PDI has a significant impact on the physical stability of nano carriers and should be kept to a minimum for long-term stability. The degree of particle size heterogeneity is less if the PDI value is closer to zero (Surini et al., 2019). The PDI values of TTO-loaded NLC were in the range of 0.147–0.345, indicating a relatively narrow size distribution. Based on the low PDI, it was found that TTO favors homogenous NLCs (Bunacuroroso et al., 2021). Among the various formulations, NLC 3 showed the narrowest particle size distribution range (0.1477 ± 0.1).

The electrostatic or charge repulsion or attraction between particles in a suspension is measured by the zeta potential. Zeta potential is a key indicator of the stability of a dispersion system since it offers in-depth knowledge of the factors that contribute to flocculation, aggregation, and dispersion. According to Leonyza and Surini (2019), a good suspension has a zeta potential value that is more positive than +30 mV or more negative than -30 mV. Five formulas of TTO-loaded NLCs initially showed a negative surface charge because the system had a negatively charged lipid (Kaur et al., 2015, Surini et al., 2020), as evident

from the zeta potentials in the range of -12.5 to -14.7 mV. The negative surface charge formed an electrical barrier on the particle's surface, which, through the repulsion mechanism, contributes to the physical stability of the nanodispersion (Patil et al., 2016). The liquid to solid lipid ratio did not show a significant effect on zeta potential. In this study, nonionic surfactant Poloxamer 188 and Span 80 were used to stabilize NLC formed. Nonionic surfactants form a space barrier, lowering the solution's zeta potential (Manzar et al., 2020). Although nonionic surfactants have low zeta potential, they tend to stabilize the formulation as a result of steric stabilization (Pivetta et al., 2018).

Thermal analysis

Fig. 1 presents the DSC thermograms of stearic acid and TTO-loaded NLCs when heated to 100°C with a 20°C increase per minute. The DSC result in Fig. 1, provided that stearic acid melted at 58.16°C, with a melting enthalpy of -171.16 J g⁻¹. The onset temperature, melting point and endothermic enthalpy of TTO loaded NLC were smaller than those of stearic acid and decreased from NLC 1 to NLC 5 with increasing amount of Miglyol 812 in the formula (Table 3). Miglyol 812 is a medium chain triglyceride, which is

used as a liquid lipid component in the developed TTO loaded NLC formula. Miglyol 812 interacts with the crystalline lipid matrix of stearic acid, which causes disruption of the crystal lattice so that the lipid becomes more amorphous after forming NLC. The higher the amount of Miglyol 812 in the formula, the lower the crystallinity of the system formed.

Similarly, according to Harisa and Badran (2015), adding Miglyol 812 to the formula lowered the melting point and crystallinity of the NLCs produced. This corresponds to a previous study that found mixing olive oil and cocoa butter with the NLC formulas caused less-ordered nanostructuring of the lipid matrix (Fatemeh Nahr et al., 2018). These findings are also in agreement with Severino et al (2011), which demonstrated that the melting enthalpy and crystallization decreased as the amount of oil in the formulation increased (inversely proportional). In a less-ordered crystalline structure, the structure's space capacity is not confined, creating enough space where drug molecules can be accommodated and, consequently, increasing the drug loading capacity (Galvão et al., 2020).

X-ray diffraction (XRD) profiles

XRD profiles explain the way lipids are arranged, phase behavior, and characteristics of lipid structures (Fatemeh Nahr et al., 2018). The intensity of sharp peaks at 21.3° NLC has decreased compared with stearic acid, meaning that the crystal structure of stearic acid changed after the liquid lipid was added (Fig. 2). Moreover, XRD peaks at 21.3° decreased from NLC 1 to NLC 5, associated with an increase in the liquid lipid-to-solid lipid ratio. This result may indicate that NLC 5 has a less regular structure than NLC 4, NLC 3, NLC 2, and NLC 1. Therefore, the addition of liquid lipid reduces the peak intensity (Fatemeh Nahr et al., 2018).

The less regular crystalline structure and the amorphous state will contribute to higher loading capacities and stability of nanocarriers (Fatemeh Nahr et al., 2018). In addition, a matrix with less-ordered sequence has more cavities in its structure. This way, an active ingredient can be introduced in a large amount and retained during storage, thus allowing a controlled release.

Terpinen-4-ol assay results

Fig. 3 shows the assay results of terpinen-4-ol as a tea tree oil marker of the prepared NLCs. The terpinen-4-ol concentration in all formulations slightly decreased from their initial concentration, 2%. It is suggested that the lowering of terpinene-4-ol concentration is affected by the manufacturing temperature

that induces tea tree oil evaporation during the process. Terpinene-4-ol is the main component of TTO, which has an evaporation rate of up to 98% in 4 hours at 30°C (Cross et al., 2008). The loss of the terpinene-4-ol component due to exposure to heat during the manufacturing process is in line with research by Salim and Eisa (2017), which states that heating is essential to accelerate evaporation of essential oil and evaporation rate of essential oil was increased with an increase of temperature from 60 to 100°C .

Stability study

During the six-month storage at 27 and 4°C , there were no significant changes in organoleptic properties, polydispersity index and zeta potential in all NLC formulations. The particle size of NLC slightly increased when stored for six months at 27 and 4°C . The increase in particle size during storage can be caused by slight agglomeration of the particles dispersed in the NLC or due to homogenous agglomeration growth. The agglomeration that occurs can be attributed to the low zeta potential value of the system, which is below -20 mV. In general, a zeta potential value that is more positive than $+30$ mV or more negative than -30 mV are acceptable characteristics to obtain improved stability (Surini et al., 2020). The obtained zeta potential in this study indicates a low nanoparticle electrostatic repulsion, thus slightly preventing the aggregation. In this study, NLC 3 showed the smallest and insignificant increase in particle size compared to other formulas. Therefore, in terms of physical stability among all TTO-loaded NLC formulas, NLC 3 is considered as the best formula.

Fig. 4 shows terpinen-4-ol concentrations in the nanostructured lipid carriers during six months of storage at 27°C and 4°C . The stability of terpinen-4-ol content meet the criterion (80-120%) when stored for six months in all formulas. There is no effect of liquid to solid lipid ratio on the chemical stability of terpinen-4-ol concentrations in TTO-loaded NLC on storage at temperature 27 and 4°C for 6 months. These results indicate that the NLC component such as stearic acid and Miglyol 812 affects the ability of NLC to protect the TTO. This study supports evidence from previous observations reporting that the solid lipid ingredients can protect the drugs against harsh environmental situations (Ghasemiyeh and Soliman, 2018).

CONCLUSION

TTO-loaded NLC with good physicochemical characteristic and stability was successfully developed using high shear homogenization method. The TTO-loaded NLCs prepared have an average droplet size of 200–400 nm, with a relatively short-range ordered

crystalline structure and a partially crystalline state based on the differential scanning calorimetry (DSC) and X-ray diffraction results. The ratio of the liquid to solid lipid has an influence on the physicochemical properties of TTO-loaded NLC, especially on particle size and crystallinity. Furthermore, the particle size slightly increased but there was no significant difference in chemical stability after six months of storage at refrigerator temperature ($4 \pm 2^\circ\text{C}$) and room temperature ($27 \pm 2^\circ$). In conclusion, based on the result of physicochemical stability study, the formula with ratio liquid to solid lipid 25:95 (NLC 3) is considered as the best formula.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Fitriani EW	Avanti C	Rosana Y	Surini S
Concepts or ideas	x	x	x	x
Design	x	x	x	x
Definition of intellectual content	x	x	x	x
Literature search	x			x
Experimental studies	x			
Data acquisition	x			
Data analysis	x			x
Statistical analysis	x			x
Manuscript preparation	x			x
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

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