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Research Paper

Quercetin Solid Lipid Microparticle Stability and Deposition in Rat Lungs: A Study of Surfactant Effect

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Abstract

This study aims to determine the effect of surfactant concentration on Quercetin SLM as a potential carrier of respiratory diseases, especially ones resulting from oxidative stress injury. Quercetin is a natural antioxidant with high activity. SLM was formed with 5% compritol 888 ATO as a lipid and different concentrations of poloxamer 188 as a surfactant. SLM was produced by a combination of emulsification and sonication involving freeze drying. SLM is characterized by organolepsis, morphology, yield, particle size, drug loading, and entrapment efficiency. The antioxidant activity of quercetin SLMs was tested using the ABTS method. SLMs are characterized as having round and smooth morphology, high yield (F1 88.53%; F2 91.44%; F3 92.87%); particle size (F1 1.81 μm; F2 1.90 μm; F3 1.94 μm); high drug loading (F1 15.96%; F2 13.74%; F3 13.19%); and high entrapment efficiency (F1 96.53%; F2 87.94%; F3 87.48%). Increasing surfactant concentration did not produce a significant difference between formulas. Quercetin SLM showed high antioxidant activity (Quercetin 94.43%; F1 94.35%; F2 94.36%; F3 94.37%). SLM was stable at storage temperatures between 25°C and 40°C. The effect of surfactant can be seen on particle size, drug loading, and entrapment efficiency at 40°C. Results of in vivo deposition study indicated that all SLM formulas were able to deliver quercetin to the lungs. Increasing the concentration of surfactant in Quercetin SLMs made no difference to the lung deposition as confirmed by observations conducted at 1 hour and 4 hours. Quercetin SLM has the potential for lung delivery by inhalation.

Keywords

Inhalation, Solid Lipid Microparticle, Quercetin, Stability, Deposition, *In vivo*

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1. INTRODUCTION

The lungs constitute the primary organs of the respiratory system. In executing their function, the lungs are continuously exposed to oxygen rendering them susceptible to harm resulting from oxidative stress (Mehta et al., 2018). Approximately 2% of the oxygen inhaled produces Reactive Oxygen Species (ROS) which causes damage to proteins, lipids, and DNA (Rogers and Cismowski, 2018). Under normal circumstances, oxidative reactions are inhibited by various natural antioxidants present in the lungs such as glutathione, superoxide dismutase, β-carotene, vitamins C and E, uric acid and heme-oxygenase. However, under certain conditions, such antioxidant protection can be overcome by reactive oxygen or nitrogen species (Mehta et al., 2018). Unfortunately, relatively few treatment options are available in cases of lung tissue damage. The primary ones are corticosteroids administered orally or through inhalation, and high-dose oral N-acetylcysteine. These treatments have limited benefits, given their significant side effects. Therefore, natural antioxidants, i.e., phytoconstituents such as flavonoids which constitute polyphenol compounds (Mehta et al., 2018) are considered. One widely used type of flavonoid is Quercetin while others, including rutin, hesperidin, and naringenin, have also been employed 3.

Quercetin (3,5,7-trihydroxy-2- (3,4-dihydroxyphenyl) -4Hchromen-4-one) is one of the secondary metabolites of plants in the form of polyphenols that belong to the flavonoid group and are found in various foodstuffs. Numerous studies have confirmed the biological activity of quercetin including one of its roles as an antioxidant (Kim et al., 2013). The total antioxidant activity of quercetin plasma is known to produce up to 6.24 times more antioxidant than Trolox which is used as a reference since it contains almost the same amount of

antioxidant as Vitamin C (Yang et al., 2020).

The therapeutic application of Quercetin is problematic due to its low solubility in water of approximately 0.0003 mg/mL; high lipophilicity (log$P$>1.8); and low oral bioavailability (2%). The absorbs are largely influenced by the nature and type of sugar attached and quercetin molecules containing 3' and 4' hydroxyl substituents with catechol groups undergo extensive methylation *In vivo* (Mehta et al., 2018, Francesca et al., 2021). This leads to frequent use of high-dose quercetin to achieve optimal therapeutic efficiency and can produce significant side effects. Therefore, to overcome this problem, non-invasive lipid-based drug delivery systems such as SLM are used (Mehta et al., 2018). Solid Lipid Microparticles (SLMs) are non-porous, micrometer-sized particles whose use promotes the incorporation of bioactive compounds and crystallization as a means of modifying the physical properties of the lipid system (Queiros et al., 2020). Solid Lipid Microparticles (SLMs) are biocompatible, biodegradable, demonstrate *In vitro* and *In vivo* bioaffinity, and can be introduced via several routes including oral, topical, nasal, pulmonary, and parenteral (Scalia et al., 2015). Visualization of an SLM is shown in Figure 1.

With regard to lung treatment, a further inhalation route is selected. A DPI (dry powder inhaler) has more advantages than nebulizers and metered dose inhalers (MDIs) because it is more practical, propellant-free, requires neither the reconstitution of dry powder nor cold chain storage, is easier to use, more stable, can be administered in large doses, and can physically accommodate novel delivery systems (Mehta et al., 2018).

Lipids and surfactants, as essential components of SLMs, play an extremely important role because they will affect the properties of their delivery system (Scalia et al., 2015). Lipids function to form the hydrophobic core of SLMs at both room and body temperature (Scalia et al., 2015), while surfactants function as particle stabilizers (Nahum and Domb, 2021). The stabilization mechanism of surfactant particles rests on their ability to reduce surface tension which can affect various characteristics of SLMs (Ameya, 2017). The selection of surfactant concentrations requires investigation because they can affect the physical stability of SLMs and it is generally determined by considerations of maximum safety and stability (Scalia et al., 2015).

Few studies of solid lipid microparticles encapsulating Quercetin have investigated inhalation drug delivery with the optimum particle size of 1 to 5 μm or focused on investigations of surfactant which lead to issues of safety, the stability of SLMs, and in vivo deposition in the lungs. Several studies observed Quercetin in the form of liposomes, nanocrystal, inclusion complexes or solid lipid nanoparticles (Kakran et al., 2012). Scalia et al. (2013) studied Quercetin SLMs with a fine particle percentage of 20.5%, less than 4.46 μm in size. Approximately 22% crossed the Calu-3 monolayer during a 4-hour period with no evidence of deposition in the lung (Scalia et al., 2013). Therefore, the aim of this study is to optimize the use of quercetin in the SLM system as a potential carrier, in the form of a dry inhalable powder, to treat respiratory system diseases. This study comprehensively compared the amount of surfactant used to evaluate the characteristics of SLMs, antioxidant activity, stability, and in vivo deposition in the lung.

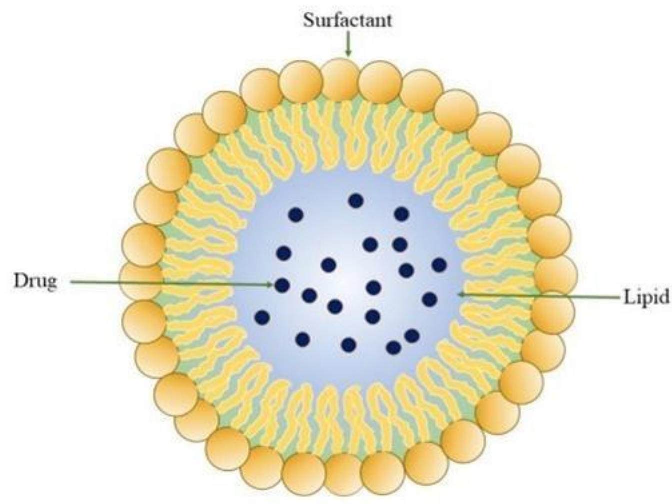

Figure 1. Visualization of a Solid Lipid Microparticle (SLM)

2. EXPERIMENTAL SECTION

2.1 Materials

The Quercetin hydrate (Tokyo Chemical Industry, Co. Ltd, Japan), Compritol ®888 ATO (National Formulary (NF)-Glyceryl Behenate) (Gattefosse, France), Poloxamer 188 (Lutrol® F68) (BASF, Ludwigshafen, Germany), methanol (Merck, Darmstadt, Germany), ABTS and potassium persulfate (Sigma Aldrich, USA) used in this research were of pharmaceutical grade.

Table 1. Quercetin Solid Lipid Microparticle Formula

Materials	Function	Formula		
		F1	F2	F3
Quercetin	Active Agent	1.0%	1.0%	1.0%
Compritol 888 ATO	Lipid	5.0%	5.0%	5.0%
Poloxamer 188	Surfactant	0.5%	1.0%	1.5%

2.2 Preparation of SLMs

SLMs were prepared using a combination of melt emulsification and sonication methods. Poloxamer 188 was dissolved in 100 mL aquadest. Poloxamer 188 and Compritol 888 ATO were heated over a hotplate to a temperature of 750°C±2°C. Once the compritol 888 ATO had melted, it was combined with quercetin until the mixture was homogeneous. Poloxamer 188 was poured on Compritol 888 ATO at a temperature

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Table 2. Antioxidant-Sample Composition

Sample	Quercetin (μL)	Quercetin SLM (μL)	ABTS (mL)	Water (μL)	Total Volume (mL)
Quercetin SLM	-	50	5	50	5.1
Quercetin	50	-	5	50	5.1
Blank	-	-	5	100	5.1

of 750°C±2°C. The emulsion formed was stirred at 10,000 rpm for three minutes using an ultra-turrax dispersing machine (Ultra-Turrax T25 IKA, Wilmington, NC) before being sonicated for two minutes (Ultrasonic Bath, Thermo Fisher Scientific Inc.). The emulsion was cooled at room temperature until a suspension of microparticles formed. The samples were freeze dried (BUCHI Lyovapor L200, Switzerland) at -50°C for 96 hours. The SLM formula is shown in Table 1.

2.3 FTIR Analysis

Approximately 10 mg of the raw material, quercetin SLM Formula 1, Formula 2, and Formula 3 were placed on the FTIR lens and the infrared absorption subsequently observed (ALPHA II FTIR Spectrometer). The spectra obtained were compared with ones contained in the reference library (Departemen Kesehatan Republik Indonesia, 2020).

2.4 Moisture Content

Moisture content was tested using a Mettler Toledo Moisture Analyzer HB43-S (Leicester, UK).

2.5 Morphology

Morphological evaluation was performed with a Scanning Electron Microscope (SEM) (FEI Type: Inspect-S50). Before being analysed, the sample was coated with a gold layer to a thickness of up to 15 nm in an argon atmosphere. Samples were dispersed on carbon plates and observed at random locations (Scalia et al., 2015). The sample was observed at four magnifications of 1500×, 2500×, 5,000×, and 10,000×.

2.6 Particle Size

Particle size was determined using a Scanning Electron Microscope (SEM) with imageJ software and an optical microscope (Optilab Biological Microscope Novel Model XSZ -107 Series®) with optical lab software. Using the optical microscope, measurements were completed on 300 particles at 400× magnification. The largest and smallest particle sizes of the sample were both determined, before being divided into several classes and intervals. The average diameter value was also identified (Sinko, 2016).

2.7 Yield

Yield was determined by comparing the weight of the Quercetin SLMs formed with the weight of each Quercetin SLM forming material, namely quercetin, compritol 888 ATO, and poloxamer 188. The percentage yield (%) was calculated using the following Equation (1) (Queiros et al., 2020):

$$\text{Yield} = \frac{\text{Weight of SLM Quercetin made}}{\text{Weight of SLM Quercetin Raw Materials}} \times 100\% \quad (1)$$

2.8 Drug Loading and Entrapment Efficiency

Approximately 50 mg of sample was added to exactly 20 mL of methanol. The sample was stirred with a magnetic stirrer for two hours before being centrifuged at 2,500 rpm for ten minutes. The supernatant was then analysed by UV spectrophotometry at a wavelength of 369 nm. The concentration of quercetin was determined, while drug loading and entrapment efficiency were calculated using the following Equations (2) and (3) (Scalia et al., 2015):

$$DLs = \frac{\text{Weight of drug in SLM}}{\text{Weight of SLM}} \times 100\% \quad (2)$$

$$EE = \frac{\text{Weight of drug in SLM}}{\text{Weight of SLM}} \times 100\% \quad (3)$$

2.9 Antioxidant Activity

As shown in Table 2, antioxidant activity was evaluated *In vitro* by means of the ABTS/TEAC (Trolox Equivalent Antioxidant Capacity) test. The working solution was prepared by mixing 7.0 mM ABTS and 2.45 mM potassium persulfate and left overnight (12-16 hours) in a dark room at ambient temperature. Under such conditions, ABTS changed to $ABTS^{\bullet +}$ which was then dissolved in 30 mL of aquadest used for preparation according to the composition shown in Table 16. Absorbance was read at a wavelength of 734 nm using a UV spectrophotometer, Quercetin SLMs were tested for six hours after preparation, whereas Quercetin was tested post-preparation for 30 minutes. The percentage antioxidant activity of Quercetin SLMs was measured using the following Equation (4) (Talarico et al., 2021):

$$\text{Scavenging}(\%) = \left(1 - \frac{\text{A Blank}}{\text{A Sample}}\right) \times 100\% \quad (4)$$

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2.10 Evaluation of Stability

Stability evaluation of Quercetin SLMs was conducted by means of accelerated stability testing. Quercetin SLMs were divided and inserted into two vials, one being stored at room temperature (25±2)°C and the other at (40±2)°C, RH 75±5% for 28 days. Quercetin SLMs were characterized according to their particle size, drug loading, entrapment efficiency, and antioxidant activity on days 0, 7, 14, 21, and 28. Quercetin SLMs were compared to Quercetin with regard to antioxidant activity. In addition, SLM was characterized according to its morphological structure on days 0 and 28 (Benke et al., 2019, Mangal et al., 2019, Kakran et al., 2012).

2.11 Drug Deposition in the Lung

Drug deposition in the lungs of Wistar rats was conducted in accordance with the method proposed by Hariyadi et al. (2021) Wistar rats which satisfied the inclusion criteria served as the research subjects. Animal experiments were performed in strict adherence with current animal handling protocols which had been granted clearance by the Ethics Committee of Universitas Airlangga. Ketamin euthanizing agent was used in this research. Quercetin doses administered as a supplement in humans fall within the range of 250-600 mg per day (Kumar et al., 2017). The amount of SLMs given to the rats was calculated from drug loadings. Administration of SLMs by means of nose-only exposure was adopted from (Kaur et al., 2008). SLMs were labelled with rhodamine B and inhaled in the previous manner explained above. The rats were euthanized and their lungs (the left and right lung caudal lobes) were excised. Observation of drug deposition was carried out using a Fluoresence Microscope (FS × 100, Olympus) fitted with a red filter at a magnification of 42× and an exposure time of 1/1.2 ms.

2.12 Data Analysis

Statistical data analysis was performed on data relating to characteristics and stability tests using the one-way ANOVA method with a confidence degree of 95% (α = 0.05). Drug deposition was observed by means of a fluorescent microscope with the intensity of the groups being subsequently compared. Qualitative and quantitative data analysis was carried out by comparing the intensity of fluorescence in the tracheal and pulmonary tissue of rats using ImageJ software. The red luminescence intensity data obtained was then compared statistically by means of a Kruskal-Wallis non-parametric analysis.

3. RESULTS AND DISCUSSION

3.1 Characterization of Quercetin Solid Lipid

Microparticles: Fourier Transform Infrared The FTIR spectra analysis results can be seen in Figure 2 and Table 3. The FTIR spectra of Quercetin SLM Formula 1, 2, and 3 contain specific function group wave numbers of the raw material, namely quercetin, compritol 888 ATO, and poloxamer 188. There is no shift in the specific group wave numbers in the formula indicating the absence of a chemical interaction between the raw material and the SLM system that had been expected to form.

3.2 Moisture Content

The results of Quercetin SLM moisture content analysis (Table 4) were encouraging (<5%) where low moisture content can prevent particle aggregation. In cases of high moisture content, accelerated agglomeration can be caused by strong interlocking forces resulting from fluid bridges between particles. This has a prejudicial effect on the flow properties and on particle size which increases considerably (Jung et al., 2018). In addition, high moisture content affects stability in storage which can culminate in both water plasticization and instability (Shetty et al., 2020). Analysis involving the use of a Kruskal-Wallis test at sig 0.441 > 0.05 indicates there was no significant difference between the SLM Quercetin formulas.

3.3 Morphology

The morphological evaluation results of Quercetin SLM Formulas 1, 2, and 3 using SEM can be seen in Figure 3. The three formulas showed morphology in the form of spherical particles and smooth surface. This finding is in line with those of several studies that reported smooth and spherical SLM (Queiros et al., 2020).

3.4 Drug Loading

Drug loading is important in enabling the evaluation of drug delivery system potential and is expected to be high in order to reduce the number of administered preparations. The drug loading evaluation results are contained in Table 4. One-way ANOVA analysis obtained sig 0.003 < 0.05 which confirms significant differences between the three formulas. The difference found in the case of Formula 1 was greater than Formulas 2 and 3 which may be due to an imbalance in the ratio of surfactants and lipids in the latter two formulas. Given excessively large amounts of surfactant, drug loading will decrease due to the strong interaction between the drug and surfactant (Wang et al., 2016).

The increase in surfactant concentration causes the drug to become more soluble in the water phase causing it to become more partitioned in that phase than in the lipid phase which is the hydrophobic core of solid lipid microparticle. Drugs partitioned in the water phase cannot enter the hardened lipid phase at room temperature with the result that the drug will be concentrated on the outer skin of the SLM which remains liquid and/or on the surface of the particles. Moreover, the amount of drug loading in Formula 1 can occur due to differences in the quantity of additives used. Of the three formulas, Formula 1, which will theoretically have higher drug loading in the drug loading calculation formula, contains the lowest amount of additives.

3.5 Entrapment Efficiency

In line with drug loading, entrapment efficiency is also expected to produce high results. SLMs with high loading lead to a reduction in the number of particles needed to achieve effective drug

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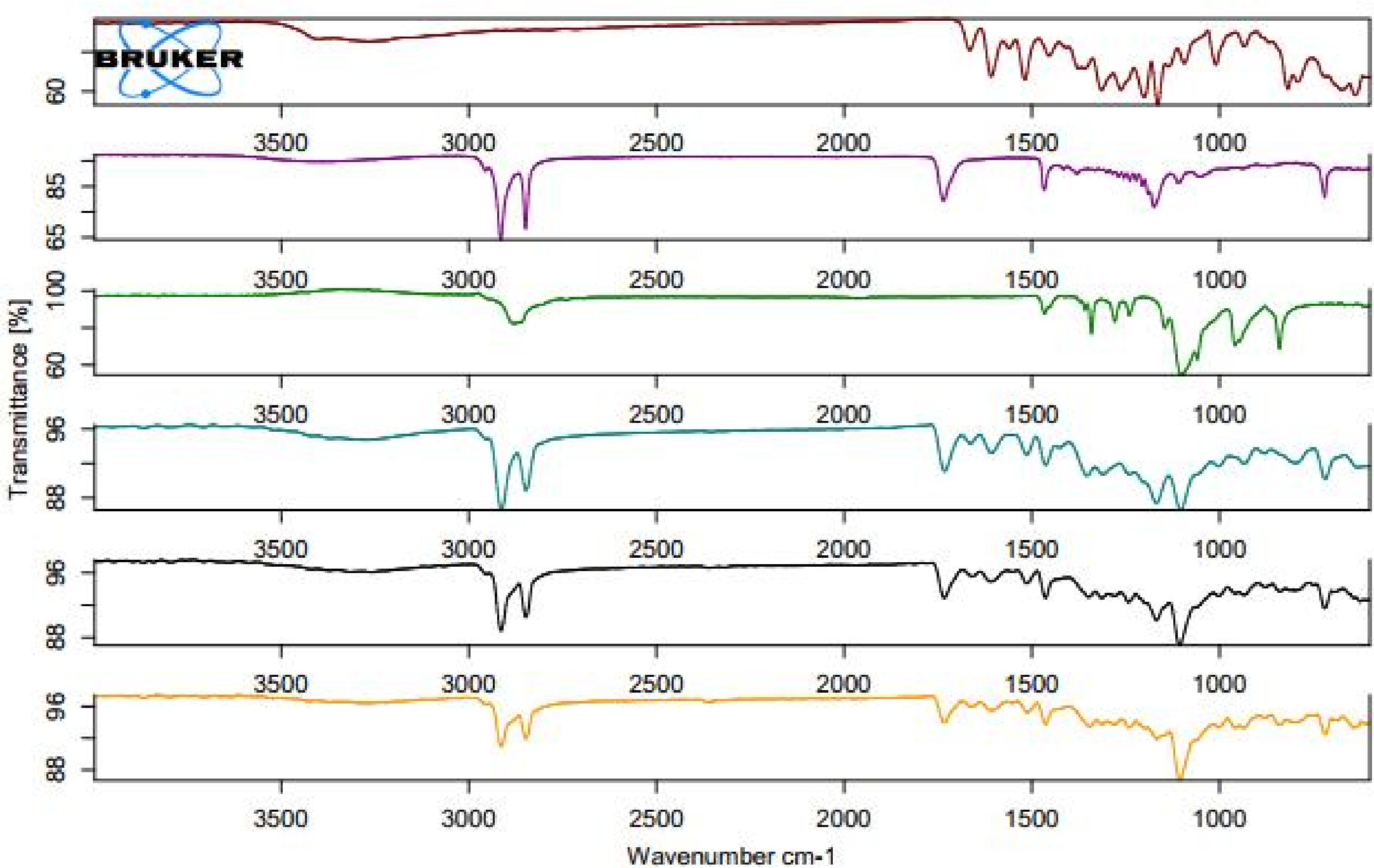

Figure 2. IR Spectra of Raw Material Overlay and Quercetin SLM Formula. Red: Quercetin, Purple: Compritol 888 ATO, Green: Poloxamer 188, Blue: Quercetin SLM Formula 1, Black: Quercetin SLM Formula 2, Yellow: Quercetin SLM Formula 3

Table 3. FTIR Raw Materials and Quercetin SLM Formula

Functional Groups	Wave Number (cm^{-1})					
	Quercetin	Poloxamer 188	Compritol 888 ATO	F1	F2	F3
−OH str.	3262.53	-	3285.54	3274.95	3262.87	3270.75
C−H str	-	2878.43	2846.78	2847.02	2847.21	2847.30
C=O str	-	-	1732.78	1732.00	1733.46	1733.54
−CH bend	-	-	1463.23	1463.10	1463.39	1463.03
O−H bend	-	1340.22	-	1353.04	1348.62	1347.08
C−O str	-	1100.92	-	1103.66	1106.29	1105.74

Table 4. MC, Yield, DL and EE of Quercetin SLM

Formula	MC (%)	Yield	Drug Loading (%)	Entrapment Efficiency (%)
1	1.83±0.63	92.87±3.15	15.96±0.87	96.53±7.31
2	1.16±0.21	91.44±4.86	13.74±0.31	87.94±5.95
3	2.01±1.46	88.53±3.80	13.19±0.54	87.48±2.67

concentrations (Scalia et al., 2015). The results for Quercetin SLM Formula 1, 2, and 3 are impressive (Table 4) indicating that the SLM preparation method can produce efficient entrapment. Data analysis involving the use of a Kruskal-Wallis test was carried out and obtained sig 0.393 > 0.05 meaning there was no significant difference between the Quercetin SLM

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Table 5. Particle Size of Quercetin SLM Formula 1, 2, and 3 by SEM Method and Optical Microscope

Formula	SEM method		Optical Microscope Method	
	Particle Size (μm)	PDI	Particle Size (μm)	PDI
1	2.48	0.17	1.81±0.20	0.14±0.06
2	2.51	0.16	1.90±0.15	0.15±0.04
3	2.31	0.16	1.94±0.25	0.14±0.02

Table 6. Quercetin SLM Particle Size after 28-day Storage at 25°C and 40°C

Temperature	Formula	Day 0 (μm)	Day 7 (μm)	Day14 (μm)	Day21 (μm)	Day28 (μm)
25°C	1	1.81±0.20	1.81±0.11	1.83±0.18	1.69±0.04	1.97±0.14
	2	1.90±0.15	1.86±0.08	2.00±0.04	1.99±0.15	1.85±0.10
	3	1.94±0.25	1.90±0.05	1.91±0.15	1.95±0.16	1.88±0.26
40°C	1	1.81±0.20	1.80±0.21	1.82±0.26	1.85±0.08	1.78±0.15
	2	1.90±0.15	2.00±0.13	2.07±0.17	1.88±0.07	2.16±0.09
	3	1.94±0.25	1.94±0.04	1.82±0.22	1.95±0.22	1.84±0.11

Table 7. Quercetin SLM Moisture Content at 40°C

Formula	Day 0 (%)	Day 7 (%)	Day 28 (%)
1	1.83±0.63	1.76±0.87	1.48±0.14
2	1.16±0.21	1.18±0.72	1.16±0.34
3	2.01±1.47	1.51±0.42	1.40±0.30

formulas.

3.6 Yield

Yield is one of the parameters that needs to be considered in addition to the purity and quality of the delivery system. Yield is expected to produce optimum results which signify the achieving of good delivery system results (Patel et al., 2019). The results indicated that the high yield of the three formulas was close to 100%, indicating that the SLM preparation method could efficiently produce the maximum amount of SLM. A one-way ANOVA statistical analysis obtained sig 0.444 > 0.005 which meant there was no significant difference between the three formulas. The effect of excipients in confirming the successful formation of other drug delivery systems by yield such as microspheres has been investigated (Hariyadi et al., 2018).

3.7 Particle Size

The particle size in all three formulas using SEM method produced a result of 2.31-2.51 μm (Table 5). After a Kruskal-Wallis statistical analysis indicated sig 0.030 < 0.05, which meant a significant difference existed between the formulas, there was a difference in F3 which, of the three formulas, had the smallest particle size. This was due to the higher surfactant content of poloxamer 188 resulting in a smaller particle size because it can more efficiently prevent particle coalescence (Ignjatovic et al., 2021). Using the optical microscope method, smaller results of 1.81-1.94 μm (Table 5) were obtained than with the SEM method. Statistical analysis using Kruskal-Wallis produced sig 0.390 > 0.05 which means there was no significant difference between the three formulas. The particle size of the three formulas in both the SEM and optical microscope methods had an expected number of 1-5 μm. Particles with a diameter of 1-5 μm can reach the lungs (Akdag, 2019). The PDI of the three formulas in both methods produced a result of <0.3 indicating the particle size of the three homogeneously distributed formulas (Danaei et al., 2018).

3.8 Antioxidant Activity of Quercetin Solid Lipid Microparticles

Based on the results obtained for three Quercetin SLM formulas, quercetin showed high antioxidant activity (>94%) (Figure 4). Quercetin SLM is expected to demonstrate this characteristic and, therefore, can act as a natural antioxidant that protects the lungs against oxidative stress (Mehta et al., 2018). Data analysis of antioxidant activity was carried out using a Kruskal-Wallis test with sig 0.057, sig > 0.05 being obtained as the results. Therefore, it demonstrated that no significant difference existed between the three formulas and quercetin.

3.9 Stability of Quercetin Solid Lipid Microparticles: Morphological Stability

Quercetin SLM morphology was observed on the 28th day of storage at 40°C. Quercetin SLM morphology on day 28 (Figure 5) was not seen to differ from that on day 0 (Figure 3) in any of the three formulas. The post-stability organoleptic results of the Quercetin SLM between day 0 and day 28 were similar remains consisting of dry, light yellow, odourless, free-flowing powder. Furthermore, the SEM-identified morphology indicated that Quercetin SLM remains spherical with a smooth surface. These organoleptic stabilities during a 28-day period are in accordance with results of particle stability where no aggregation occurs and the ability to maintain high antioxidant activities is evident. This could be due to the stable SLM organoleptically resulting in a high concentration of Quercetin which is protected inside the solid lipid microparticles.

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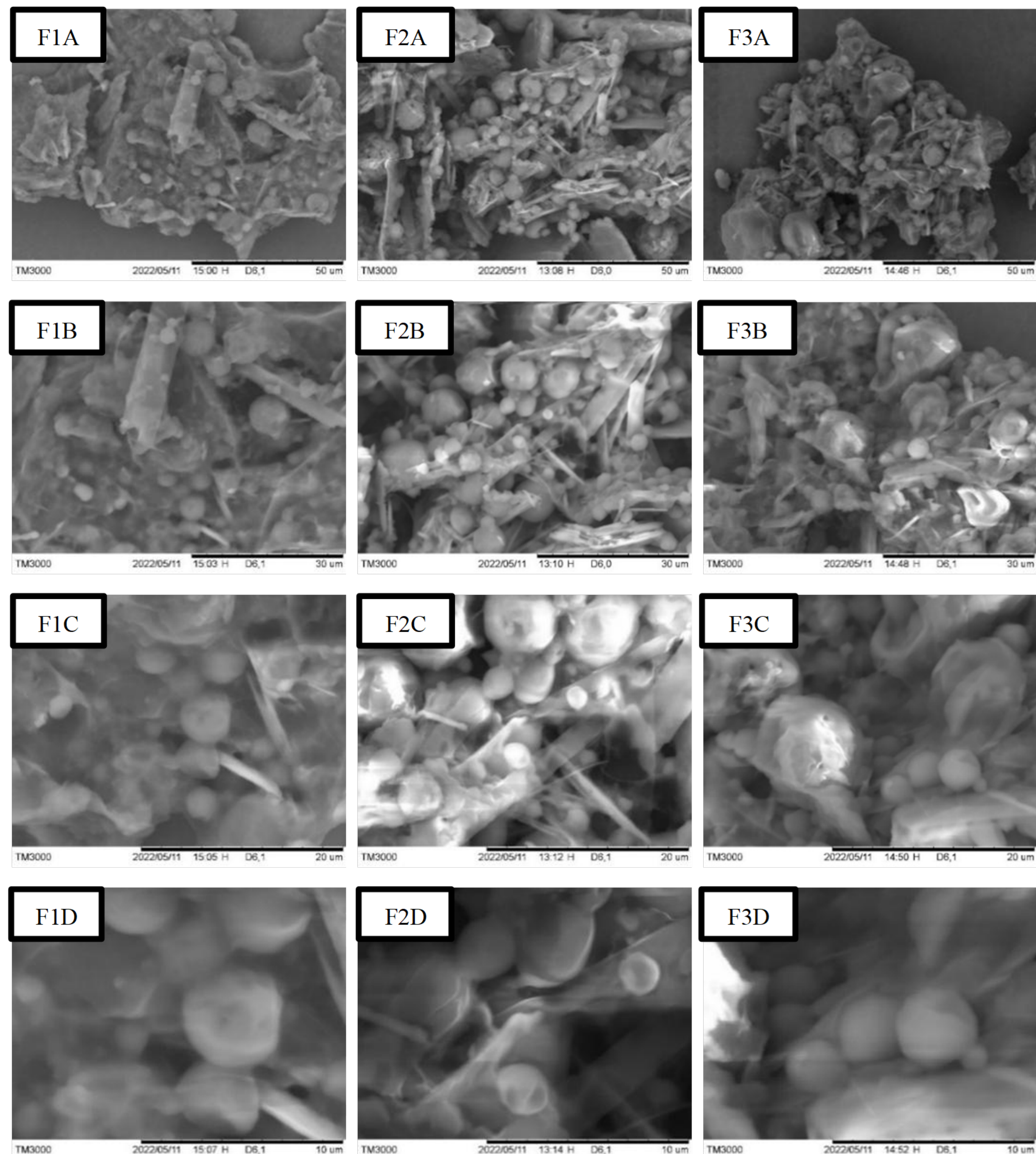

Figure 3. Quercetin SLM Morphology of Formula 1 (F1), Formula 2(F2), and Formula 3 (F3) at A: 1,500×, B: 2,500×, C: 5,000×, D: 10,000× Magnification

3.10 Particle Size Stability

Particle size stability in storage represents a valid topic of study in DPI samples due to the possibility of changing the particle size from the optimum range to enable access to the lungs (1-5 μm) (Benke et al., 2019). The particle size stability was

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Table 8. Stability of Quercetin SLM Drug Loading after 28-day Storage at 25°C and 40°C

Temperature	Formula	Day 0 (%)	Day 7 (%)	Day 14(%)	Day 21(%)	Day 28(%)
25°C	1	15.97±0.88	14.72±0.56	14.75±0.73	15.42±1.43	15.08±2.15
	2	13.73±0.31	12.62±2.41	13.76±0.94	12.60±1.93	13.57±0.89
	3	13.19±0.54	11.93±2.04	11.59±1.76	11.79±1.68	12.82±1.05
40°C	1	15.97±0.87	16.20±2.84	16.72±0.87	15.74±1.59	15.23±1.07
	2	13.73±0.31	14.23±1.80	13.95±1.78	12.31±0.28	13.62±1.80
	3	13.19±0.54	12.62±1.30	13.68±1.34	11.80±1.57	11.27±2.76

Table 9. Entrapment Stability Efficiency of Quercetin SLM after 28-day Storage at 25°C and 40°C

Temperature	Formula	0 day (%)	Day 7(%)	Day14 (%)	Day21 (%)	Day28(%)
25°C	1	96.53±7.31	88.95±4.74	93.26±5.81	93.32±11.17	91.28 ±15.10
	2	87.94±5.95	81.04±18.41	88.25±10.63	80.68±13.75	86.84±7.29
	3	87.48±2.67	78.82±10.60	76.62±8.90	78.60±14.73	85.05±6.22
40°C	1	96.53±7.31	97.87±17.34	95.12±12.06	95.30±12.63	92.04±7.28
	2	87.94±5.95	91.46±16.35	89.61±15.14	78.77±4.26	87.10±12.10
	3	87.48±2.67	83.71±7.56	90.73±8.38	78.43±11.65	75.14±20.14

Table 10. Stability of Antioxidant Activity of Quercetin SLM at 28 Days of Storage at 25°C and 40°C

Temperature	Formula	0 day (%)	Day7 (%)	Day14 (%)	Day21 (%)	Day28 (%)
25°C	Quercetin	94.43±0.00	95.81±0.02	97.60±0.01	98.13±0.00	96.4±0.01
	1	94.35±0.04	95.74±0.04	97.59±0.01	98.13±0.01	96.39±0.02
	2	94.36±0.02	95.74±0.03	97.59±0.01	98.13±0.01	96.38±0.02
	3	94.37±0.02	95.7±0.10	97.59±0.01	98.13±0.01	96.38±0.03
40°C	Quercetin	94.43±0.00	95.58±0.04	97.55±0.00	98.09±0.00	96.33±0.00
	1	94.35±0.04	95.44±0.04	97.56±0.01	98.1±0.01	96.34±0.01
	2	94.36±0.02	95.45±0.07	97.56±0.01	98.1±0.01	96.34±0.00
	3	94.37±0.02	95.47±0.08	97.57±0.01	98.1±0.00	96.34±0.01

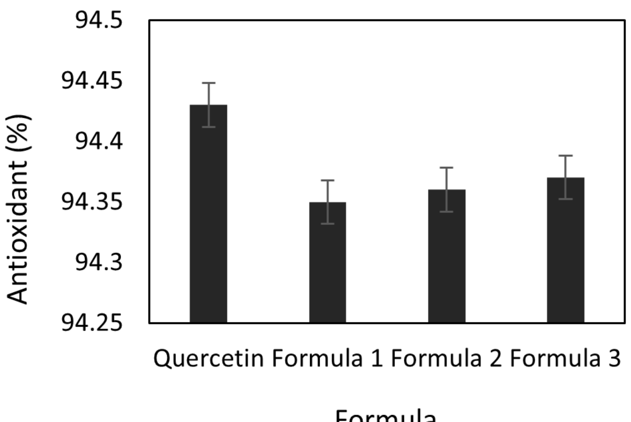

Figure 4. Antioxidant Activity of Quercetin and SLM Quercetin

measured in this study using an optical microscope, with the results presented in Table 6. At both storage temperatures, the particle size remained, as expected, within the range of 1-5 μm

Table 11. Drug Deposition Results for Tracheal Tissue

Formula	Average Intensity	Standard Deviation
Formula 1	506.352	179.974
Formula 2	916.540	397.388
Formula 3	1046.224	541.776

Table 12. Drug Deposition Results in the Left Lung

Formula	Average Intensity	Standard Deviation
Formula 1	5804.545	2659.294
Formula 2	5640.509	1878.692
Formula 3	3441.982	2852.523

(Akdag, 2019). Statistical analysis of particle size stability at a storage temperature of 25°C was performed on the formula and day by means of two-way ANOVA. Against the formula, sig 0.147 > 0.05 was recorded which means there was no significant difference between the three formulas. Against the day, sig 0.944 > 0.05 was recorded which means there was no significant difference between days, thereby indicating that Quercetin SLM is stable at particle size parameters given a

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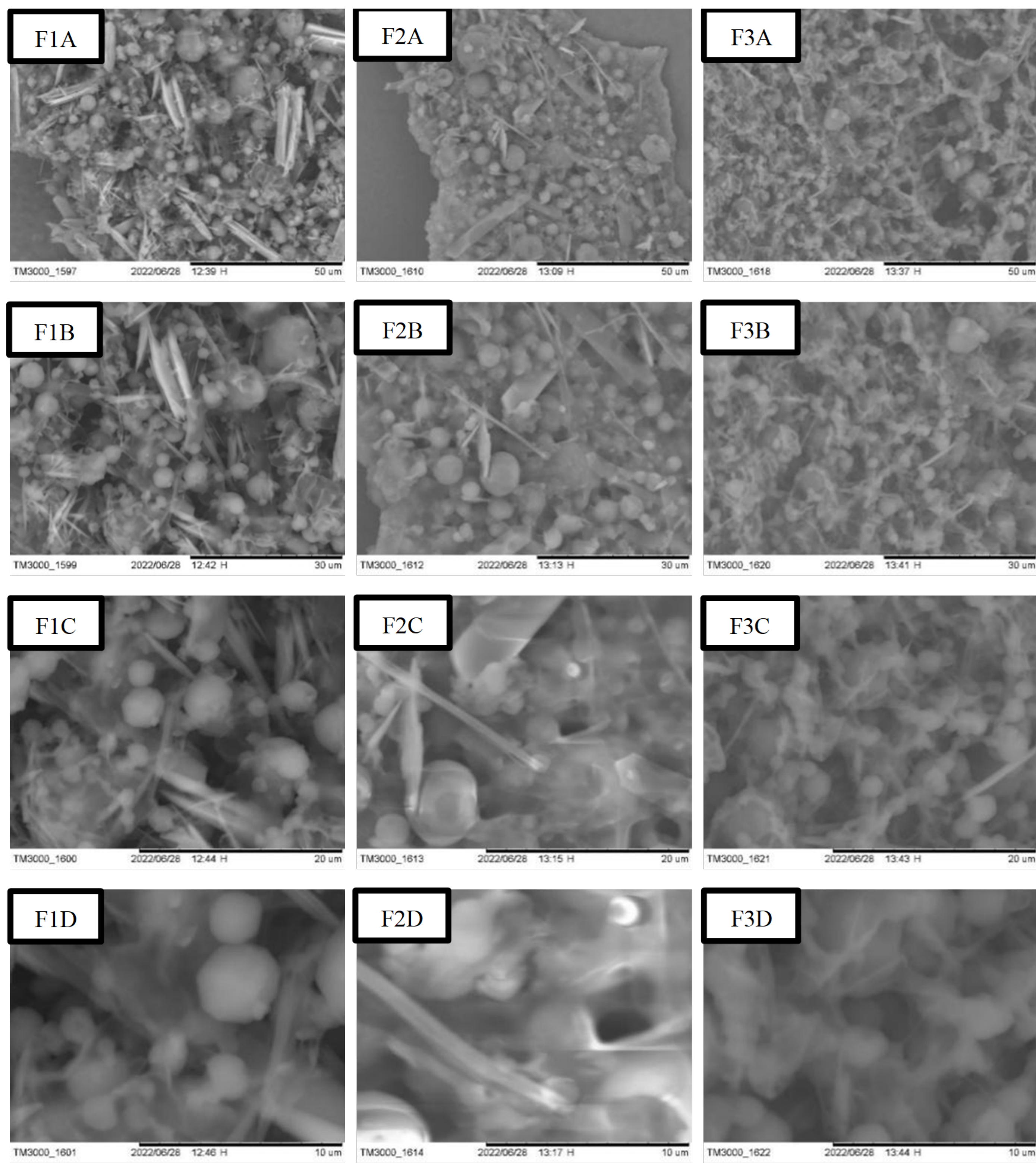

Figure 5. Quercetin SLM Morphology Day 28 Formula 1(F1), Formula 2 (F2), and Formula 3 (F3) at Magnification A: 1,500×, B: 2,500×, C: 5,000×, D: 10,000× Magnification

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Table 13. Comparison of Drug Deposition in Tracheal Tissue

	Red filter	Overlay
Formula 1		
Formula 2		
Formula 3		

storage temperature of 25°C. At a storage temperature of 40°C, the particle size remained stable (sig 0.990) and the specific formula exerted an influence while in storage (sig 0.016) as shown by the significant difference between Formulas 1 and 2. This difference can be caused by sample moisture influenced by storage temperature. The moisture content of Formula 1 tended to decrease while in storage, whereas that of Formula 2 remained relatively fixed (Table 7). With a decrease in moisture content, the particle size of Formula 1 became smaller, differing significantly from Formula 2 which did not experience a similar reduction.

3.11 Drug Loading Stability

The stability evaluation of Quercetin SLM drug loading is shown in Table 8. Statistical analysis of drug loading stability was performed using two-way ANOVA on the formula and day. This test indicated that drug loading remained stable at both storage temperatures (Sig 0.403 at 25°C and Sig 0.205 at 40°C). The stability of drug loading demonstrated a significant statistical difference between formulas (sig 0.000), with the difference being found in Formula 1. This can be caused by the initial drug loading data (drug loading on day 0) since the level initially found in Formula 1 was significantly different to that of the other two formulas.

3.12 Stability of Antioxidant Activity

The stability of antioxidant activity remained high in storage (see Table 10). Statistical analysis of antioxidant activity stability was performed using two-way ANOVA of formula and day. The stability of antioxidant activity had a significant difference to the formula (Sig 0.003 at 25°C and Sig 0.004 at 40°C) and the difference lay in the quercetin sample. Quercetin experienced the greatest decrease in antioxidant activity of all three Quercetin SLM formulas. This corresponds to the advantages of a solid lipid microparticle drug delivery system in terms of the stability of the preparation. SLM has a solid matrix that encapsulates the active ingredient and protects it against degradation (Scalia et al., 2015). The stability of antioxidant activity differed significantly between days (Sig 0.000 at 25°C and Sig 0.000 at 40°C) due to the $ABTS^{\bullet+}$ reagent used which rendered it unstable in storage. Even with the same method of preparation, Reagent $ABTS^{\bullet+}$ recorded different absorbances at each measurement taken at the specified time interval. In this case, ABTS should be adjusted until a certain absorbance of 0.7 or 1.1 (Shah and Modi, 2015). Moreover, differences can be caused by the ABTS method which has disadvantages

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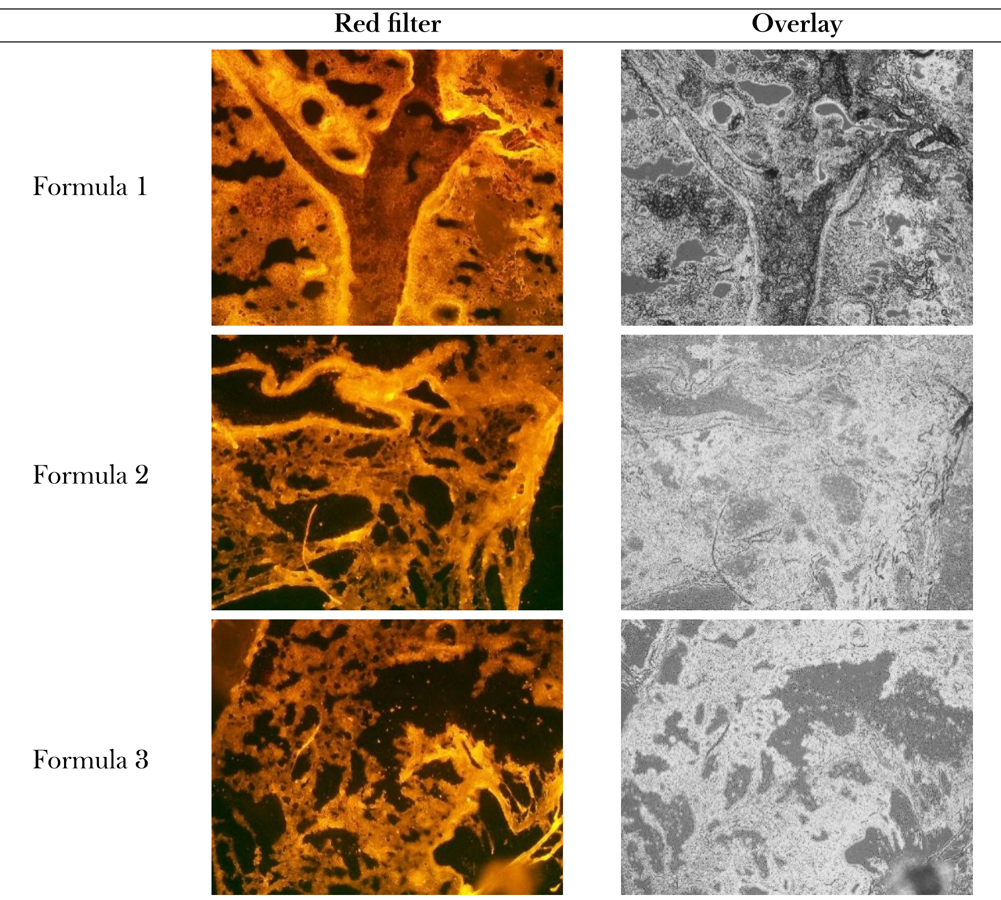
Table 14. Drug Deposition Comparison in Left Lung



in terms of radical instability which means that results cannot be reproduced (Shah and Modi, 2015).

3.13 Entrapment Efficiency Stability

Stability entrapment efficiency showed a high yield in storage (Table 9). Statistical analysis of entrapment efficiency stability was performed using two-way ANOVA of formula and day. At a storage temperature of 25°C, sig 0.367 > 0.05 is obtained against the formula which means there was no significant difference between the three formulas. Meanwhile, against the day obtained sig 0.427 > 0.05 which means there was no significant difference between days confirming that SLM Quercetin was stable at the entrapment efficiency parameter at a storage temperature of 25°C. At a storage temperature of 40°C, entrapment efficiency remained stable (sig 0.483). The effect of the formula was apparent in the significant difference between formulas, Formula 1 being greater than Formula 3. This difference can be caused by the degradation occurring in the surfactant. The poloxamer 188 used as surfactant can degrade at a storage temperature of 40°C and a relative humidity of 75% for six months (Chen et al., 2022). Degradation of the surfactant caused quercetin to escape from the carrier system and resulted in a decrease in entrapment efficiency. Formula 3, containing the largest amount of surfactant, was more affected by the degradation of the surfactant than Formula 1 which contained the smallest amount.

3.14 Quercetin SLM Deposition Test: Fluorescence Imaging

The deposition test for SLMs on the lungs was carried out one hour after sample administration by observing the presence, or otherwise, of fluorescence from Rhodamin B added to SLMs. Observation conducted one hour after sample administration is in keeping with previous research undertaken by Azizah et al. (2019) where, at that point, rhodamine B fluorescence was shown to be present in the rats' lungs (Azizah et al., 2019). The observation was conducted at the Center for Stem Cell Research and Development, Universitas Airlangga, using the Inverted Fluorescence Microscope (Olympus CKX53). Observation of the trachea and the caudal lobes of both lungs with a fluorescence microscope was performed at a magnification of 40×.

The deposition test results showed that the increase in surfactant concentration in the SLM formula did not affect the drug deposition results in the lungs of rats. However, it should be noted that rhodamine B was not bound to the active ingredi-

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Table 15. Comparison of Drug Deposition in the Caudal Lobe of the Right Lung

	Red filter	Overlay
Formula 1		
Formula 2		
Formula 3		

Table 16. Measurement of Rhodamine B Concentration in the Lungs

Formula	Rhodamine B Concentration (μg/g)			
	Left Lung		Right Lung	
	1 hour	4 hours	1 hour	4 hours
1	1.13±0.41	0.90±0.31	0.71±0.12	0.61±0.14
2	6.34±8.36	2.06±1.93	14.73±24.21	0.90±0.43
3	2.95±3.01	3.08±2.62	0.63±0.29	2.81±3.14

ent quercetin and had slightly different properties to quercetin. Consequently. rhodamine B fluorescence cannot perfectly explain the presence of quercetin. Therefore, further research is necessary using other fluorochromes that can effectively describe the active agent.

3.15 Deposition Test Performed on Tracheal Tissue

Observation results relating to deposition tests performed on tracheal tissue can be seen in Table 13. The results of a calculation relating to fluorescence intensity in tracheal tissue subsequently performed using the ImageJ application can be seen in Table 11.

These results confirmed that each formula produced fluorescence in the tracheal tissue. The fluorescence intensity in Formula 1was lower than those of Formulas 2 and 2, possibly due to the comparatively smaller particle size in that formula. As a result, the SLM particles in Formula 1 were not retained to any great extent in the trachea. The results of the analysis using one-way ANOVA showed a significance of 0.301 (sig > 0.05). Therefore, there was no significant difference in fluorescence intensity between the formulas relating to tracheal tissue.

3.15.1 Deposition Test on Left Lung

The observation results of the deposition test on the left lung can be seen in Table 14. These results indicate that each formula produces fluorescence in the left lung (Table 12 and Table 14). The fluorescence intensity of Formula 1 was greater than those of Formula 2 and 3, while the intensity of Formula 3 had

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the lowest value. This result could be caused by the smaller size of the particles in Formula 1 relative to the other formulas with the result that more SLM particles in Formula 1 were deposited in the lungs. The ANOVA-one way analysis produced a significance result of 0.478 (sig > 0.05) indicating that there was no significant difference between formulas in terms of fluorescence intensity in the left lung. The fluorescence intensity was then calculated using the ImageJ application. The results of the calculation of fluorescence intensity in the left lung can be seen in Table 12.

3.15.2 Deposition Test on Right Lung

The observation results of the deposition test on the caudal lobe of the right lung can be seen in Table 15. The fluorescence intensity was subsequently calculated using the ImageJ application. The results of the calculation of fluorescence intensity in the caudal lobe of the right lung can be seen in Table 13.

These results indicate that each formula produces fluorescence in the caudal lobe of the right lung (Table 13 and Table 15). The fluorescence intensity in Formula 1 had a greater value than those of Formulas 2 and 3, while the intensity of Formula 3 was the lowest. This result could be caused by the smaller size of the Formula 1 particles compared to those of other formulas leading to more SLM particles in Formula 1 being deposited in the lungs. The results of the one way-ANOVA analysis were significant at 0.198 (sig > 0.05). Therefore, there was no significant difference in fluorescence intensity between formulas in the caudal lobe of the right lung.

3.16 Quantitative Test

A Quercetin SLM deposition test was quantitatively carried out by calculating the concentration of rhodamine B added in SLM. The concentration of rhodamine B was calculated using regression equations at the maximum wavelength using UV-vis Spectrophotometry. Determination of the standard solution's concentration is based on the minimal absorbance of the homogenate sample of the pulmonary organs. The linear regression equation between concentration and absorbance produced the result y= 0.3089x – 0.0122 with a correlation coefficient value r of 0.9992. The coefficient value is greater than in the t table with a confidence degree of 95% (α = 0.05) which shows a linear relationship between the concentration of the standard solution and absorbance. Furthermore, lung homogenate was determined using UV-VIS Spectrophotometry at a wavelength of 551.5 nm and lung homogenate blank without treatment. The concentration of rhodamine B in the lungs (μg/g) was calculated using the previously applied rhodamine B equation. The following is the concentration of rhodamine B in the lungs after 1 hour and 4 hours of treatment of the caudal lobes of the left and right lungs (Table 16).

From the data in the Table 16 it can be seen that experimental animals administered with Formula 2 samples had high rhodamine concentration values in both the left and right lungs. However, after analysis using two-way ANOVA, a sig value of 0.383 was obtained in the left lung and a sig value 0.432 (sig > 0.05) in the right lung. Therefore, there was no significant difference between the formulas.

Experimental animals administered with Formula 1 and 2 experienced a decrease in rhodamine B concentration in the lungs between one and four hours after treatment. This showed that when SLMs had contained the drug for the 4th hour, Rhodamine B was less present than at the one hour point. Although anomalies existed, for example in Formula 3, the concentration of rhodamine at the 4-hour point was higher than in the first hour, the increase was not significantly different. This was because a two-way ANOVA, produced sig values of 0.438 in the left lung and 0.421 (sig > 0.05) in the right lung with no significant difference between the 1st. and 4th. hour of treatment. These results show that SLM can maintain the presence of active agents in the lungs, a fact confirmed by no significant decrease in rhodamine concentration having occurred after four hours of treatment. However, to corroborate the data obtained, it is necessary to conduct deposition testing with a longer treatment time and a negative control (rhodamine B without SLM encapsulation) to confirm how long SLM is able to maintain the active agent in the lungs and its profile when compared to a negative control. In addition, a deposition test using fluorochrome which can be conjugated with the active agent or has similar properties to quercetin, such as lipophilic nile red, should be carried out in order to confirm the presence of the active agent in the lungs.

4. CONCLUSIONS

This study examines Quercetin SLM as a potential carrier of airway was successfully produced with high yield, drug loading, and entrapment efficiency, thus promoting manufacturing efficiency. Quercetin SLMs demonstrate suitable size for airway inhalation high antioxidant activity, making them suitable as a natural antioxidant for respiratory tract problems. The optimum formula represents the highest drug loading, high yield and entrapment efficiency, low MC, good morphology and particle size, stability during storage and high antioxidant activity. Quercetin SLMs was able to deliver quercetin to the lungs. The increase in surfactant concentration did not induce a difference in the deposition in the lungs after 1 hour and 4 hours of treatment. The MMAD evaluation (aerodynamic median mass) is further recommended for inhalation delivery system.

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