Short Communication:

Analytical Method Validation and Formula Optimization of Topical Nanoemulsion Formulation Containing Resveratrol

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Abstract: *Resveratrol (RSV), a natural lipophilic phytoalexin, was reported as an antioxidant and anti-inflammatory agent, which has the potential to cure diabetic wounds. However, several studies suggested the limitation of RSV, such as poor aqueous solubility, poor stability, and poor oral bioavailability. To overcome the issues, RSV was formulated as a topical nanoemulsion. It is important to ensure the quality of the dosage form by evaluating RSV load in the nanoformulation and optimizing the formula. A reversed-phase HPLC method was developed and validated prior to the load determination of RSV in the nanoemulsion formulation. The composition of triacetin-eugenol, Kolliphor® RH 40, and Transcutol® was further optimized by employing a Box-Behnken Design (BBD) to achieve the optimum composition with expected viscosity and RSV load. The HPLC method for determining RSV load was successfully validated for parameters of selectivity with the resolution of 8.487, linearity and range (r = 0.9979), precision (0.12% of RSD), accuracy (109–110% of recovery), the limit of detection (0.574 µg/mL), and limit of quantitation (1.740 µg/mL). The result of formula optimization was promising, showing the optimum composition of triacetin-eugenol, Kolliphor® RH 40, and Transcutol® at 4.44 g, 30.97 g, and 11.39 g, respectively.*

Keywords: *Box-Behnken design; formulation; nanoemulsion; resveratrol; validation*

■ INTRODUCTION

A diabetic wound is one of the severe complications in diabetic patients with uncontrolled blood glucose levels. The slow healing in a diabetic wound is likely due to endothelial malfunction and the blocked angiogenesis [1]. Further severities as gangrene infection and tissue death lead to amputation surgery in up to 50% of the diabetic ulcer wound cases [2-3]. Many studies have been done to discover natural chemicals which can cure diabetic wounds. There has been increasing evidence that resveratrol (RSV) plays an important role in promoting skin healing in diabetic wounds, for instance, by activating SIRT 1 pathway and potentially upregulating nuclear Nrf2 and Mn-SOD [1,4].

Resveratrol (3,4′,5-trihydroxystilbene; RSV) is a natural lipophilic phytoalexin richly extracted from grape skin, red wines, berries, and edible peanut [5-8]. Besides promoting skin healing on wound treatment, RSV is also therapeutically claimed to reduce the risk of cardiovascular diseases, diabetes, and neuro-disorders due to its potent antioxidant and anti-inflammatory properties. [6,9-15]. RSV also supports collagen activity and can act as UV photo-protection [16-17].

Apart from the potential therapeutic benefits of RSV, as a lipophilic antioxidant, the aqueous solubility of RSV is very limited, and RSV is prone to chemical instability [4,18]. The oral administration of RSV is also challenging, reflecting poor bioavailability due to excessive hepatic first-pass elimination [19]. Therefore, to be more effective in diabetic wound treatment, topical administration with the capability of promoting the aqueous solubility, protecting the RSV stability, and enhancing the acceptability is the most appropriate strategy in delivering RSV.

Various topical RSV nanoformulation studies have been previously reported [20-22], including a formulation of self-assembly topical nanocarriers developed by Nastiti et al. [8]. The nanocarriers, which were further called nanoemulsions, were emulsion-based systems containing oil phase, surfactant, cosurfactant, and water and showing excellent clarity in appearance. The system enabled RSV to be nanoscale-dispersed in the formulation. The study [8] also demonstrated good evidence that the nanoemulsion formulation maintained the stability of RSV in an extended time of storage. Various formulas had been well established in this study to achieve the target of clear and stable RSV nanoemulsion, which showed expected skin penetration. However, there was a lack of information on the effect of the components on the physical characteristic of the nanoemulsion.

The RSV load and viscosity are two important properties in the characterization of RSV nanoemulsion. The RSV load on each formula must be examined to ensure whether the content of the RSV in the nanoemulsion is uniform and met the specification, despite the composition variation of the other components. Viscosity plays an important role in determining the consistency and "the ability to spread" of the nanoemulsion. Those indicate the ease and convenience of application, which becomes another aspect of the quality of topical nanoemulsion. Besides, viscosity determination provides important information to overcome consistency-handling issues with regards to formulation, manufacturing, and packaging [23]. The varied intrinsic viscosity of the nanoemulsion components such as oil phase, surfactant, and cosurfactant, as well as their composition, may affect the viscosity of the nanoemulsion system. Therefore, it is imperative to evaluate the RSV load and to observe the effects of the composition of the oil phase, surfactant, and cosurfactant on the viscosity of nanoemulsion. Optimization is also important to be carried out to develop RSV nanoemulsions with better quality.

Box-Behnken Design (BBD) is one of the optimization methods recently used in the nanoemulsion formulation area [24-27]. BBD allows the formulators to optimize more than two factors of formulation in three different levels to obtain multiple response optimization [28-29]. BBD has a distinct advantage compared to the central composite design (CCD) in a way that BBD requires fewer number of points; therefore, it is more economical and more efficient [30].

This current study demonstrated BBD-based optimization of RSV nanoemulsion developed based on the previous literature [8]. The responses observed were viscosity and RSV load. A simple reversed-phase HPLC method was successfully developed and validated prior to the determination of the RSV. The method referred to an HPLC method developed by Dwiastuti et al. [31] in analyzing 4-n-butyl resorcinol in the nanosystem formulation. An open-source R software package called "rsm" was also applied to economically implement a BBD model for computer-aided optimization purposes [29].

■ EXPERIMENTAL SECTION

Materials

RSV was purchased from PCCA (99% purity, USA). Solvents used in this study were acetonitrile (gradient grade for liquid chromatography, Merckmilipore) and redistilled water (PT. Ikapharmindo Putramas, Indonesia). Topical nanoemulsion was prepared in the Faculty of Pharmacy, Sanata Dharma University, Indonesia, according to the previous study [8] with some modifications. Triacetin, eugenol, Kolliphor® RH 40 (polyoxyl 40 castor oil), and Transcutol® (ethoxydiglycol) were purchased from Sigma-Aldrich (Singapore).

Instrumentation

Instrumentation used in this study were HPLC system of Shimadzu® LC-2010 CHT coupled with UV/Vis detector, C_{18} column of Apollo® (150 × 4.6 mm i.d, 5 µm), Scaltec® SBC 22 analytical balance (max. 60/210 g, min. 0.001 g), Retsch® T460 ultrasonicator, sterile syringe filter (0.2 µm pore size) hydrophilic PTFE membrane (Merck millipore), Gast® vacuum pump model DOA-P504-BN, and a set of Socorex® micropipettes. Response surface methodology and response optimization were performed using R Studio software version 1.1.456 with rsm packages [32]. All

formulations were self-assembly supported by a magnetic stirrer (Thermo, China). The viscosity of the nanoemulsion was measured by using a Merlin VR Viscometer (Rheosys, USA).

Procedure

Standard solutions for the RSV assay

Stock solution (SS) was made by dissolving 100 mg of RSV standard into 10 mL of solvent and diluting it to achieve the concentration of 100 μg/mL in acetonitrile-water (50:50). SS was further diluted to prepare a series of concentrations in a range of 10–40 μg/mL for standard solutions.

HPLC condition

An HPLC method using Shimadzu® LC-2010 CHT with LabSolution software and UV-VIS detector was applied. An Apollo® C18 column of Grace Davison Discovery Sciences (150 × 4.6 mm i.d, 5 μm) was used. An isocratic elution system was developed with a composition of mobile phase containing acetonitrile-water (50:50), and the mobile phase flow rate was 0.8 mL/min. Chromatographic separation was detected at 308 nm. The sample injection volume was 10 μL, and the stop time setting was at 5 min.

System suitability test

A system suitability test for the HPLC method was carried out before conducting the analytical method validation by injecting a sample of diluted blank topical nanoemulsion, which was added with RSV to achieve the RSV concentration of 12 μg/mL. This injection was replicated six times.

Analytical method validation

HPLC method for determining the content of resveratrol was further validated for parameters such as selectivity, linearity and range, precision, accuracy, the limit of detection, and the limit of quantitation, according to the AOAC guidelines [33].

Selectivity. The selectivity test in this study was performed by injecting a solvent, standard solution, and sample solution of the diluted blank topical nanoemulsion (2000 times dilution) in the HPLC system to achieve a chromatogram profile for each solution. The selectivity parameter was assessed by evaluating the obtained resolution value of the RSV peak.

Linearity and range. Linearity and range were examined by injecting a series of standard RSV solutions in a concentration range of 10–40 μg/mL. Calibration equation and coefficient correlation were obtained to indicate the parameter of linearity and range.

Limit of detection and limit of quantitation. The sensitivity parameter was assessed by determining the limit of detection and the limit of quantitation. In this study, the determination of limit of detection and limit of quantitation was executed by injecting a series of standard RSV solutions in lower concentrations, followed by calculating the limit of detection and limit of quantitation using the standard deviation approach [34].

Accuracy and precision. Accuracy and precision were evaluated in intra-day and inter-day schemes. Samples of diluted blank nanoemulsion were added with RSV SS to achieve three different concentration levels (low, medium, and high). Each concentration was replicated three times. The accuracy parameter was derived from the recovery. A precision parameter was evaluated from the percentage of relative standard deviation (RSD) in intra-day and inter-day schemes.

Topical nanoformulation

RSV nanoemulsion is fabricated using a spontaneous emulsion method based on the formula developed by Nastiti et al. [8] with some modifications, incorporating 2% of RSV as the active ingredient. The formula of the base was arranged as shown in Table 1.

Triacetin and eugenol were mixed in the same ratio of 1:1. Kolliphor® RH 40 (surfactant) and Transcutol® (cosurfactant) were mixed in the ratio S/CoS of 3:1. All ingredients were mixed using a magnetic stirrer at room temperature.

Table 1. RSV nanoemulsion formulation

Component	Amount (g)
Triacetin-eugenol	10
Kolliphor® RH 40	30
Transcutol®	10
Citrate Buffer pH 6	50

Experimental design

BBD was applied to optimize the composition of Triacetin-eugenol, Kolliphor® RH 40, and Transcutol®. The composition of Triacetin-eugenol (X1), Kolliphor® RH 40 (X2), and Transcutol® (X3) was stated as the independent variables, whereas RSV load (Y1) and viscosity (Y2) were stated as the dependent variables. Every independent variable was coded as -1, 0, and +1 for low, medium, and high levels, respectively. Table 2 presented the coded levels of variables and their values.

Sixteen experimental runs were generated by applying BBD in an "R" software. Every single run was then made based on the method mentioned above. RSV load for each formula was determined as a dependent variable by a validated HPLC method. The viscosity of each formula was also evaluated as another dependent variable.

RSV load determination. To determine the content of the RSV in the nanoemulsion, the samples were prepared by diluting the formulation 2000 times using the mobile phase prior to injection to the HPLC.

Viscosity test. The viscosity of the nanoemulsion was measured by a Merlin VR viscometer (Rheosys, USA) with the system of cup and bob, using 25 mm concentric (co-axial) cylinders, in a rotation speed of 50 rpm. The measurement was done twice for each formula at ambient temperature, with a delay time of 10 sec and an integration time of 20 sec. The zero-shear time was set 100 sec in between the measurements.

Table 2. Coded levels of variables and the observed values in the RSV nanoemulsion

Variables	Coded levels		
	-1	0	+1
X1: Triacetin-eugenol (g)	5	10	15
X2: Kolliphor® RH40 (g)	25	30	35
X3: Transcutol® (g)	5	10	15

RSM observation. The results of BBD experimental runs were recorded and listed to generate the RSM model. Statistical analysis for RSM resulted from analysis of variance (ANOVA) report to observe the significant differences among independent variables. Desirability analysis was performed using R software to evaluate the optimized condition for further formulation process.

■ RESULTS AND DISCUSSION

System Suitability Test

The system suitability test was performed to ensure the performance of the developed analytical method. HPLC separation properties such as retention time, peak area, retention factor, resolution, tailing factor, and theoretical plate number were observed as the system suitability test parameters. Chromatographic separation profiles of RSV were depicted in Fig. 1.

Table 3 presents the results of the system suitability test. According to the results, the relative standard deviation (RSD) of retention time and the peak area have

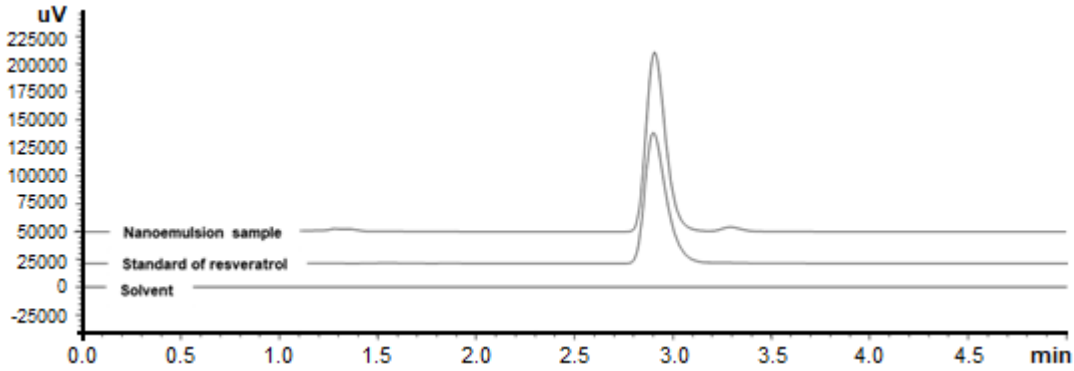

Fig 1. Representative HPLC chromatograms of acetonitrile as solvent, standard solution of resveratrol, and nanoemulsion sample solution. Mobile phase: acetonitrile-water (50:50 v/v). Flowrate: 0.8 mL/min. Column: Apollo® C18 column of Grace Davison Discovery Sciences (150 × 4.6 mm i.d, 5 µm). Wavelength detection at 308 nm. Volume of injection: 10 µL

met the requirements of RSD < 2.0 [35]. Retention factor (k), resolution (Rs), tailing factor (TF), and the number of the theoretical plate (N) also met the acceptance criteria of k > 2.0, Rs > 2, TF < 2.0, and N > 2000, respectively [36]. Hence, the system was confirmed to perform a method validation since all the parameters have met the requirements of expected analytical performance.

Analytical Method Validation

Analytical method validation was conducted in this study to confirm that the assay can be applied as intended [37]. Table 4 presents several validation parameters assessed for determining RSV content. The accuracy and precision were also evaluated, as presented in Table 5.

The assay was linear with the correlation coefficient (r) of 0.9979 (Table 4) with the linearity equation of y = 84719x – 415217, in the range of 10.01–40.04 μg/mL. The limit of detection and the limit of quantitation were 0.574 and 1.740 μg/mL, respectively. The intra-day and inter-day measured concentrations of RSV met the acceptance criteria (< 8%, Table 5). The percentage of the recovery as the accuracy parameter were also met the acceptance criteria [33] since their values were in the range of 80–115%.

Topical Nanoemulsion Formulation and Optimization

Triacetin is generally used as a low-viscosity oil phase in an emulsion-based formulation with a good solubilizing effect and good clarity [38]. Triacetin is also known as a good skin penetration enhancer [39]. Eugenol is a penetration-enhancing terpene that can be incorporated into the nanoemulsion system to increase skin penetration and permeation [40]. Eugenol also acts as an external antioxidant to protect the active ingredient from early oxidation in the formulation [41]. Kolliphor® RH 40 is a non-ionic surfactant with an excellent solubilizing effect on lipophilic compounds [42]. Kolliphor® RH 40 has been used in a wide range of oral and topical emulsion-based formulations. While the composition could produce stable nanoemulsion with an excellent solubilizing system for RSV, the viscosity of triacetin-eugenol, Kolliphor® RH 40, and Transcutol® might impact the consistency and the ability to spread of nanoemulsion in the skin. Therefore, it is imperative to optimize those factors in the RSV nanoemulsion formulation.

Table 3. Results of system suitability test (n = 6)

System suitability test parameters	Results
Mean of retention time (min)	2.940
RSD of retention time (%)	0.063
Mean of peak area	227292.833
RSD of peak area (%)	0.121
Retention factor	2.705
Resolution	8.487
Tailing factor	1.295
Theoretical plates number	3868.667

Table 4. Validation parameters for the determination of RSV

Validation Parameters	Results
Concentration range (μg/mL)	10.01–40.04
Intercept (*a*)	–415217
Slope (*b*)	84719
Correlation coefficient (*r*)	0.9979
$S_{y/x}$	13546.421
S_a	10555.286
S_b	4180.516
LOD (μg/mL)	0.574
LOQ (μg/mL)	1.740

Note: S_a = standard deviation of the intercept; S_b = standard deviation of the slope; $S_{y/x}$ = standard deviation of the residual

Table 5. Evaluation of accuracy and precision for RSV Determination

Concentration Levels	Intraday measured concentration (n = 3)			Interday measured concentration (n = 3)		
	Mean (mg/g)	Recovery (%)	RSD (%)	Mean (mg/g)	Recovery (%)	RSD (%)
Low	16.445	109.365	0.243	16.777	111.273	1.714
Medium	27.130	108.251	2.333	27.500	109.438	1.508
High	38.697	110.287	0.823	38.719	110.061	0.624

RSV topical formulations were successfully fabricated using the spontaneous method and resulted in brownish-yellow, transparent nanoemulsion (Fig. 2). The transparency has already indicated the nano-scaled size of RSV [8]; therefore, particle size analysis was not conducted.

The BBD and experimental responses, including the RSV load and the viscosity of nanoemulsion formulation, are presented in Table 6.

Experimental Design and RSM Evaluation

A total of 16 experimental runs were carried out with different combinations of variables X1, X2, and X3 and four central point replication of the second order response surface (Table 6). All the data was coded as -1, 0, and +1 for different experimental levels of the low, medium, and high, respectively. After successfully coded, RSM analysis was performed to assess the optimized composition for the nanoemulsion formula. RSM models were generated both for RSV content and viscosity. The RSM perspective plots of RSV content were depicted in Fig. 3. Three perspective plots for the RSV load model were shown with saddle properties of the surface. This revealed that the stationary point was fairly near the experimental area, but the eigenvalues were a mixed sign of positive and negative [32]. Model equation for RSV content (Y1) was $Y1 = 20.478 - 0.136X_1 + 0.575X_2 - 0.399X_3 + 1.554X_1X_2 - 0.547X_1X_3 - 1.701X_2X_3 + 2.065X_1^2 + 0.264X_2^2 - 0.503X_3^2$. The multiple R^2 and adjusted R^2 were 0.717 and 0.293, respectively. However, it should be noted that independent variables would significantly affect the responses only if multiple $R^2 \geq 0.8$ and adjusted $R^2 > 0.8$, with the difference between multiple R^2 with the adjusted R^2 must be less than 0.2 [43].

The RSM perspective plots of viscosity were depicted in Fig. 4. Three perspective plots for the viscosity model showed nearly plain saddle properties of the surface. This also revealed that the stationary point was fairly near the experimental area, but the Eigen values

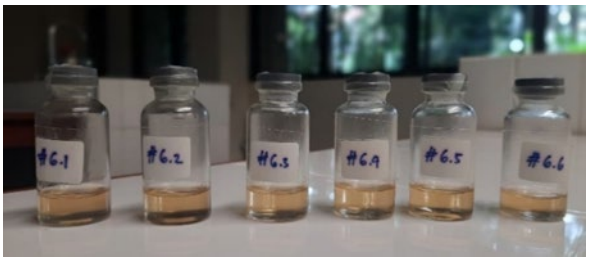

Fig 2. Representatives of optimized RSV nanoemulsion

Table 6. The Box-Behnken Design and experimental responses of RSV nanoemulsion

Run	Coded Levels			Independent Variables			Dependent Variables	
	triacetin-eugenol	Kolliphor® RH 40	Transcutol®	triacetin-eugenol	Kolliphor® RH 40	Transcutol®	RSV load (mg/g)	Viscosity (dPa.s)
1	-1	-1	0	5	25	10	25.107	0.4
2	1	-1	0	15	25	10	19.938	0.6
3	-1	1	0	5	35	10	22.569	2.4
4	1	1	0	15	35	10	23.614	2.3
5	-1	0	-1	5	30	5	19.991	1.2
6	1	0	-1	15	30	5	22.602	2.1
7	-1	0	1	5	30	15	22.572	1.1
8	1	0	1	15	30	15	22.995	1.3
9	0	-1	-1	10	25	5	19.216	1.5
10	0	1	-1	10	35	5	24.347	3.8
11	0	-1	1	10	25	15	19.533	0.7
12	0	1	1	10	35	15	17.862	2.0
13	0	0	0	10	30	10	20.221	1.7
14	0	0	0	10	30	10	20.448	1.8
15	0	0	0	10	30	10	20.448	1.7
16	0	0	0	10	30	10	20.795	1.7

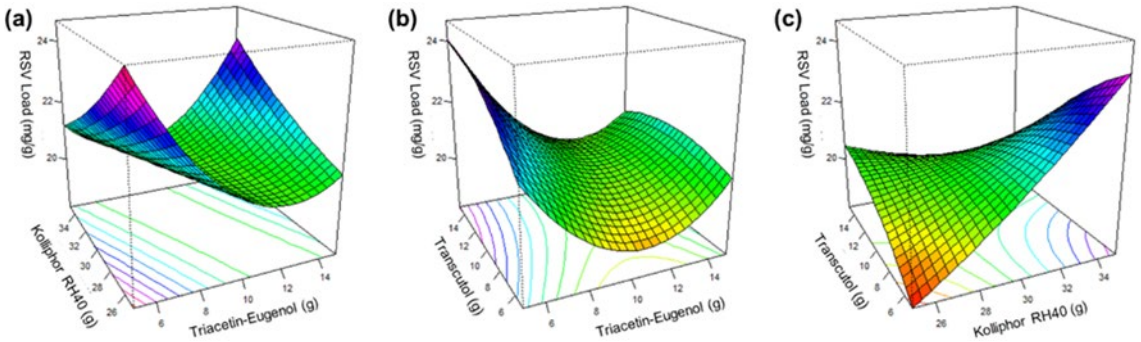

Fig 3. Perspective plots of Kolliphor® RH 40 vs. triacetin-eugenol (a), Transcutol® vs. triacetin-eugenol (b), and Transcutol® vs. Kolliphor® RH 40 (c) for resveratrol (RSV) load

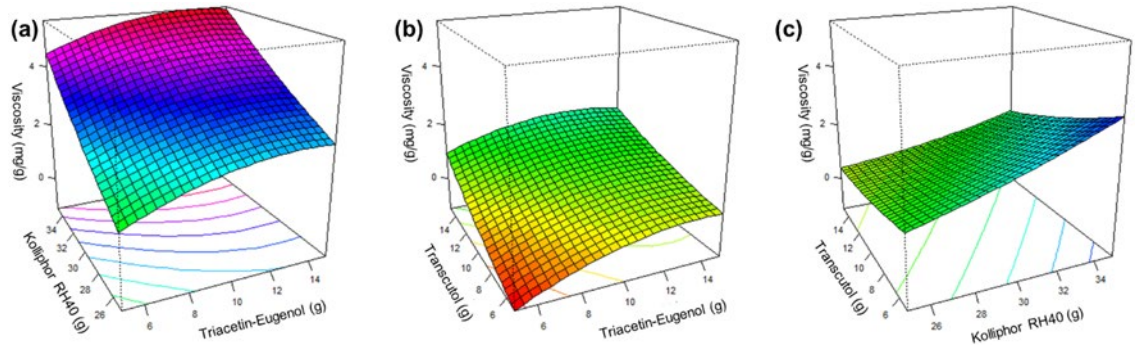

Fig 4. Perspective plots of viscosity of Kolliphor® RH 40 vs. triacetin-eugenol (a), Transcutol® vs. triacetin-eugenol (b), and Transcutol® vs. Kolliphor® RH 40 (c)

were in mixed signs of positive and negative [32]. Model equation for viscosity (Y2) was $Y2 = 1.725 + 0.150X_1 + 0.913X_2 - 0.438X_3 - 0.075X_1X_2 - 0.175X_1X_3 - 0.250X_2X_3 - 0.438X_1^2 + 0.138X_2^2 + 0.138X_3^2$. The multiple R^2 and adjusted R^2 were 0.951 and 0.878, respectively. According to these results, the response of viscosity was significantly affected by independent variables since the multiple $R^2 \geq 0.8$ and adjusted $R^2 > 0.8$, with the difference between multiple R^2 with the adjusted R^2 was only 0.073. RSV was solubilized in most of the compositions due to the surfactant and cosurfactant capacity. Therefore, the content of RSV could be completely measured with the recovery at around 100% in similar results. However, the viscosity characteristics of triacetin-eugenol, Kolliphor® RH 40, and Transcutol® are various, and the composition could lead to the various viscosity of the nanoemulsion. Hence, the viscosity data was stated as the main consideration for building a prediction model in desirability analysis.

Table 7 shows the analysis of variance for RSV load and viscosity models. Properties of the RSM model, including first-order, two-way interaction, and pure quadratic of independent variables and their errors, are presented. Desirability analysis was performed as a statistical response optimizer tool for selecting the optimum formula of nanoemulsion. Desirability calculation resulted in a value in the range between 0–1. The desirability value of 0 represents a completely unexpected response, while 1 represents the most expected response [44]. Fig. 5 illustrates the contour plot of RSV nanoemulsion formula optimization from desirability analysis. Although the RSM model for RSV content was not quite significant, the expected response for RSV content was set to the maximum value (the lowest level at 19 mg/g and targeted value at 25 mg/g) for desirability consideration, whereas the expected response for viscosity lied in the range of 1.5–2.5 dPa.s. These expected conditions were scripted in R software to

Table 7. ANOVA for RSV Load and Viscosity Models

Statistical Analysis	RSV Load					Viscosity				
	Df	Sum Sq	Mean Sq	F value	Pr (> F)	Df	Sum Sq	Mean Sq	F value	Pr (> F)
FO(X1, X2, X3)	3	4.0664	1.3555	0.4596	0.72054	3	8.3725	2.79083	33.8283	0.000372
TWI(X1, X2, X3)	3	22.4171	7.4724	2.5339	0.153301	3	0.395	0.13167	1.596	0.286166
PQ(X1, X2, X3)	3	18.3431	6.1144	2.0734	0.205246	3	0.9169	0.30562	3.7045	0.08085
Residuals	6	17.6939	2.949			6	0.495	0.0825		
Lack of fit	3	17.5256	5.8419	104.1096	0.001571	3	0.4875	0.1625	65	0.003152
Pure error	3	0.1683	0.0561			3	0.0075	0.0025		

Notes: FO= first order, TWI= two way interaction, PQ= pure quadratic; X1= triacetin-eugenol , X2= Kolliphor® RH 40, X3= Transcutol® (X3)

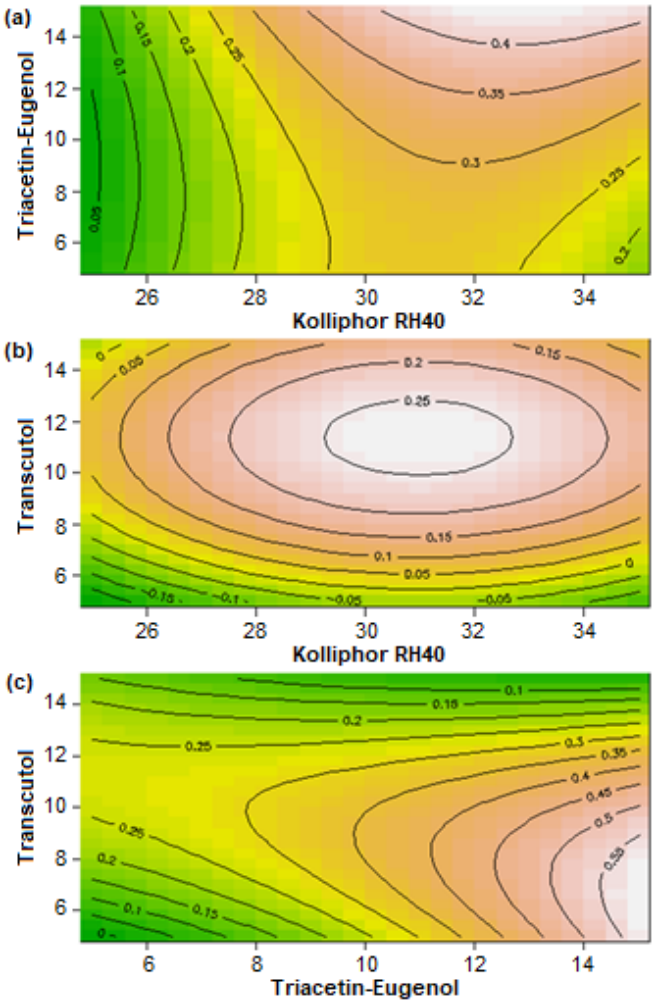

Fig 5. Contour plots of RSV nanoemulsion formula optimization according to the desirability analysis; slice at triacetin-eugenol: 4.44 g, Kolliphor® RH 40: 30.97 g, and Transcutol®: 11.39 g. (a): Kolliphor® RH 40 vs. triacetin-eugenol; (b): Kolliphor® RH 40 vs. Trascutol®; (c): triacetin-eugenol vs. Transcutol®

generate desirability analysis. It was found that the optimized compositions suggested by the RSM model were 4.44, 30.97, and 11.39 g for triacetin-eugenol, Kolliphor® RH 40, and Transcutol®, respectively; with the combined desirability was 0.346.

Several studies have been done in terms of RSV formula development and optimization. Nastiti et al. [8] reported the development and evaluation of RSV nanoemulsion intended for anti-aging skin treatment. The study explored the different compositions of excipients and penetration enhancers with the goal of the acceptable amount of RSV penetrated in the skin. The use of BBD was reported by Poonia et al. [45] to optimize the nanoemulgel containing methotrexate in combination with RSV to treat rheumatoid arthritis. Entrapment efficiency and globule size were the responses to optimize the composition of oil and aqueous phase. The optimized formulation (NEF 6 gel) was further evaluated in terms of *ex vivo* drug permeation, anti-inflammatory effect, and anti-arthritis activity and was superior to the MTX-RSV gel as the control.

This current study highlights the successful RSV nanoemulsion formula optimization with an R statistical software ("rsm" packages), an open-access software, in exploring the effect of the oil phase, surfactant, and co-surfactant on the viscosity of the formulation, which potentially affects the performance of formulations. A simple, validated assay method was also successfully developed in supporting the RSV load determination of the nanoemulsion.

■ CONCLUSION

Analytical method validation and formula optimization of topical nanoemulsion formulation containing RSV were successfully performed. The HPLC method for determining RSV content was validated and met the acceptance criteria for parameters including selectivity with the resolution of 8.487, linearity and range (r = 0.9979), precision (0.12% of RSD), accuracy (109–110% of recovery), the limit of detection (0.574μg/mL), and limit of quantitation (1.740μg/mL). A validated HPLC method was then employed to determine the RSV load in the nanoemulsion formula.

RSM model was generated by performing 16 experimental runs with three factors and three levels. The composition of triacetin-eugenol, Kolliphor® RH 40, and Transcutol® were assessed as the independent variables, while viscosity and RSV load, resulted from HPLC analysis, were evaluated as dependent variables. The BBD was also successfully applied for optimizing the composition of triacetin-eugenol, Kolliphor® RH 40, and Transcutol® in topical nanoemulsion formulation containing RSV, with predicted optimized composition of 4.44, 30.97, and 11.39 g for triacetin-eugenol, Kolliphor® RH 40, and Transcutol®, respectively.

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