ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Vol 15, Issue 9, 2022

Online - 2455-3891
Print - 0974-2441

Research Article

OPTIMIZATION, DEVELOPMENT, AND SAFETY EVALUATION OF OLIVE OIL NANOEMULSION FOR TOPICAL APPLICATION: A RESPONSE SURFACE METHODOLOGY

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Received: 20 June 2022, Revised and Accepted: 03 August 2022

ABSTRACT

Objective: Nanoemulsions consist of fine oil-in-water dispersions, with droplets covering the size range of 50–500 nm. Olive oil is frequently utilized in cosmetic and pharmaceutical topical product for its healing, protecting, and moisturizing properties due to its high fatty acid and antioxidant content. In the present work, a nanoemulsion composed of olive oil, Span 80 as surfactant, and Labrasol as cosurfactant was developed using a high-pressure homogenization method and was evaluated for its physicochemical characteristics.

Methods: Response surface methodology was utilized to investigate the influence of the main nanoemulsion components: olive oil (X_1), Span 80 (X_2), and Labrasol (X_3) on the droplet size (Y_1) and polydispersity index (PDI) (Y_2). A total of 17 formulations were generated by the Box-Behnken model.

Results: The model was found to be highly significant with R^2 values of 0.9833 and 0.9382 for droplet size and PDI, respectively. The optimized nanoemulsion presented the droplet size of 144.2±0.8 nm and PDI of 0.105±0.014. Span 80 seems to be the most influential factor that determines the droplet size as it has higher significant linear and interaction effects. The developed nanoemulsion was, further, evaluated with an *in vivo* skin irritancy study using the rat model.

Conclusion: Results indicate that the developed nanoemulsion did not demonstrate any skin irritations in gross and histological examinations, suggesting that it is safe for topical applications.

Keywords: Olive oil, Nanoemulsion, Response surface methodology, Skin irritation.

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INTRODUCTION

Skin is the largest organ of the human body as it serves as an important barrier and acts as the body's first line of defense from external physical, chemical, and biological factors [1]. Although the human skin possesses high self-regeneration potential, some skin conditions and defects might not completely heal, which can result in amputations or even death. Topical applications are usually applied directly onto the skin to maximize the penetration of active or functional ingredients into the skin with the help of a vehicle for a specific site of a body. The main advantage of topical delivery is that it can deliver drugs more selectively to the specific site. It also allows utilization of drugs with a short biological half-life and narrow therapeutic window to increase the duration of action. Moreover, it also offers several advantages over oral and injectable drug delivery, such as avoidance of hepatic first-pass metabolism, improved patient compliance, and convenient to use and apply to the skin [2]. Therefore, topical application is a suitable drug delivery system to treat skin defects. To date, there are various pharmaceutical agents such as creams, ointments, and solutions that are available for the management of skin defects. Meanwhile, Nanoemulsions (NE)-based preparations preparations are gaining much interest nowadays in the pharmaceutical, cosmetics, and biotechnology fields.

Nanoemulsions are kinetically stable or metastable formulations having nanosized droplets ranging from 50 to 500 nm. Due to their relatively small droplet size, this characteristic enhances the penetration of active ingredients or drugs [3]. The usage of nanoemulsions has been a great interest in pharmaceutical field due to their vast benefits such as improved rates of absorption and bioavailability, high loading capacity, and low skin irritation [4]. In one study, a nanoemulsion cream was found to be an efficient carrier for kojic dipalmitate (KDP) in the hair follicles, which enhances the storage behavior of the KDP in the skin, thus prolonging the activity of KDP compared to the particles of normal creams [5]. The main compositions to formulate nanoemulsions are oil, water, and surfactants or emulsifier. Surfactants are required in the nanoemulsion formulation to increase its stability by reducing the interfacial tension and stabilizing the dispersed phase against coalescence. The most popularly used surfactant or emulsifiers include polyethylene oxide-containing block copolymers, polyethylene glycol derivatives, and glycerides [4]. Meanwhile, the oil phases that are commonly used include natural or synthetic fatty acids and lipids.

Many studies proved that fatty acids could contribute to enormous advantages to the skin either applied topically or taken through the diet. Oleic acid is used topically to increase the bioavailability and delivery of poor water-soluble drugs in tablet formulations and as a raw material for ointments and creams [6]. Oleic acid also has a high penetration into the skin, with the action of altering the structure of the skin. The previous studies reported that the oleic acid applied to the skin surface of rats successfully enhances the flux of propylene glycol into the dermis and improves the stability of propylene glycol [7]. There are several types of oils that have been known to have a high oleic acids content. Olive oil is reported to be rich in monounsaturated fatty acids that account for 58–83% of oleic acid. Olive oil is a great choice as a topical product in cosmetic and pharmaceutical product due to its repairing capacity and protecting and hydrating ability when applied topically to the skin, mainly due to its high fatty acid and antioxidant content. Olive oil is also widely used as an ingredient in the production of soaps, perfumes, creams, and bath products due to its surfactive, emollient, and skin conditioning properties. It can also function as an epidermal antimicrobial barrier by forming an occlusive layer on the skin [8]. *In vivo* and *in vitro* studies have shown that olive oil phenolics have

biological activities such as altering the lipid composition, lowering the cholesterol levels, aiding cell function, and reducing oxidative damage as well as inflammation in humans and animals [9]. Topical application of virgin olive oil after sun exposure can prevent skin cancer [10]. This oil also is reported to inhibit the production of nitric oxide at the wound site [11]. Moreover, researchers have found that oleic acids promote faster wound closure compared to alpha-linolenic acids or linoleic acids.

One major limitation of using nanoemulsions as a dosage form is the stability issue that arises on their storage for long periods of time [12]. However, the kinetic balance of nanoemulsions over relatively long periods of time can be provided with the correct choice of particle size and size distribution and these factors depend on the type of oil and surfactants [13]. A pseudoternary phase diagram is generally used to determine the optimum concentration range of components, particularly for the development of nanoemulsions. However, this conventional experiment is inefficient, time consuming, and relatively costly, because it requires a large number of trials to carry out the research [14]. Moreover, parameters that influence the experiment's results must be studied independently. As a result, the interactions between many variables at a time cannot be predicted. Due to these limitations of the conventional method, a more systematic experiment design such as response surface methodology (RSM) can be implemented, so that all possible combinations of the variables or at the least, the most important ones are included in the design. RSM is a collection of mathematical and statistical techniques that are employed to model and analyze problems, in which a response (output variable) is caused by many independent variables (input variables) [15]. In RSM, a series of experiments or runs can be designed using mathematical software such as Design-Expert (Stat-Ease Inc, USA) to optimize the response by making changes to the input variables to identify the causes of changes in the response. Usually, the outcomes of the response are shown in graphical form [16]. The advantage of RSM over conventional methods is that it can simultaneously evaluate all potential input variables to develop and optimize new formulations. This means that less number of experimental runs are needed to evaluate the functional relationship between multiple variables and experimental response [17]. Having fewer experimental runs to carry out also means savings in time and resources [18].

In the present study, the concentrations of nanoemulsion components which are olive oil, surfactant (Span 80), and cosurfactant (Labrasol) were optimized and validated using RSM based on Box-Behnken design to correlate the parameters. Interaction effects between variables were evaluated using 3D contour plots. The optimized nanoemulsion which produces a small droplet size with good distribution was then subjected to characterization and skin irritancy test to ensure that the formulation is acceptable to be applied topically.

METHODS

Materials

Benzalkonium chloride, hydroxypropylmethylcellulose, (HPMC), sodium lauryl sulfate (SLS), and olive oil were manufactured by Sigma-Aldrich (USA). Span 80 was obtained from Merck (USA). Labrasol was manufactured by Gattefosse (Westwood, NJ). All other chemicals and solvents were of reagent grade and used without further purification.

Box-Behnken design for optimization of olive oil nanoemulsion formulation

The olive oil nanoemulsion was optimized using RSM. A three-factor and three-level Box-Behnken design was applied to employ the interaction of independent variables. The independent variables were the composition of olive oil (X_1 [20–70 w/w, %]), span 80 (X_2 [5–50 w/w, %]), and Labrasol (X_3 [0–25 w/w, %]), while droplet size (Y_1) and polydispersity index (PDI, Y_2) as dependent response. The Box-Behnken design generated by Design Expert software (version 6.0.6, Stat ease Inc, Minneapolis, USA) showed 17 experimental runs with five replicated center points (*) and 12 other points in random order, for which quadratic model is termed as:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X^2 + b_{22}X^2 + b_{33}X^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 \quad (1)$$

Where Y is the measured response associated with each other factor level combination; b_0 is constant; b_1, b_2, and b_3 are linear coefficients, b_{12}, b_{13}, and b_{23} are interaction coefficients among three factors, b_{11}, b_{22}, and b_{33} are quadratic coefficients of observed experimental values; and X_1, X_2, and X_3 are mentioned levels of independent variables [19]. The experiments were conducted in randomized order to minimize the effect of unexplained variability on the actual response due to extraneous factors.

Preparation of the olive oil nanoemulsion formulation

The oil phase was composed of olive oil (15.9% w/w), span 80 (9.7% w/w), and Labrasol (4.4% w/w), while the aqueous phase was of distilled water (q.s). The oil phase was fixed to aqueous phase at 30/70% w/w. The oil phase was accurately weighed and stirred. The oil components were added drop-wise to the aqueous phase and homogenized using an Ultra-Turrax T25 at 10,000 rpm for 3 min. The obtained pre-emulsion was, further, homogenized using APV 2000 high-pressure homogenizer for seven cycles at 900 bars. To thicken the formulation, 0.5% w/w of HPMC was dissolved with a sufficient amount of heated distilled water at 60°C and admixed using Ultra-Turrax T25 at 10,000 rpm for 3 min. Olive oil NE was preserved with benzalkonium chloride (0.05% w/w).

Characterization of olive oil nanoemulsion

Droplet size and PDI

The formulation (25 µL) was diluted with 25 mL of water in a volumetric flask. The flask was inverted and shaken gently to mix thoroughly. The droplet size and PDI of the resultant emulsion were determined by photon correlations spectroscopy with a Malvern Zetasizer Nano ZS, UK). This yields the mean diameter (z-average, intensity-weighted diameter) and the PDI as a measure for the width of the size distribution. The routine for the analysis was programmed so that 10 measurements were taken, 20 s per measurement and a mean was calculated eliminating the outer layers. This yields PCS data with lower standard deviations when using the built-in routine. Zeta potential was measured by determining the electrophoretic mobility using Malvern Zetasizer Nano ZS. The measurements were repeated 3 times at 25°C.

pH

Olive oil NE was measured by immersing the probe of the pH meter at a depth of 0.5 cm into the sample at 25°C. The pH meter was calibrated with standard buffers of pH 4, pH 7, and pH 10 before use. The test was repeated 3 times.

Transmission electron microscopy

The morphology of the olive oil NE formulation was observed through Tecnai G2 F20 X-TWIM TMP (FEI, Czech Republic) transmission electron microscope. One drop of the formulation was diluted with deionized water and sonicated for 15 min. The sample was loaded onto the carbon film-coated 400 mesh copper grid held with self-locking fine forceps and air-dried at room temperature before being examined under the microscope.

Animals

All of the animal studies protocols were approved by the animal ethics committee of Universiti Kebangsaan Malaysia (FF/2016/MOHD/18-MAY/751-OCT-2016-AUG-2017) and conducted according to the Care and Use of Laboratory Animals, Universiti Kebangsaan Malaysia. The study was performed on 6–8 weeks old male and female Wistar Albino rats with a body weight of approximately 200–300 g. The rats were procured from the Laboratory Animal Resource Unit, Medical Centre,

Universiti Kebangsaan Malaysia and properly housed in polypropylene cages containing saw dust as a bedding material for acclimatization. The animals were maintained under standard laboratory conditions for 7 days with controlled conditions of temperature 22±1°C and 12 h light/dark cycle before any experiments were conducted. The animals were fed with standard mouse pellet and water *ad libitum.*

Skin irritation studies

A total of 18 adult rats were used for determining skin irritation potential of the formulations according to the OECD 404 guidelines. The study was conducted on three groups of rats (six rats per group), namely, (I) normal saline as negative control, (II) 20 % w/v SLS as a standard irritant, and (III) olive oil NE. Approximately 24 h prior the initiation of experiment, the dorsal of the rats were clipped free of fur by an electric shaver. 0.1 g of sample was weighed and topically applied to the hairless skin area (3 cm^2). The area was covered with gauze, wrapped with a semi-occlusive bandage and fixed into place by non-occlusive adhesive tape. The rats were then housed one animal per cage that contained filter paper as a bedding material. Each cage was appropriately labeled. Following 4 h of exposure, the wrappings were removed. Any remaining samples were carefully wiped off with wet disposable paper towels without disturbing the integrity of the epidermis. The animals were inspected for erythema and edema at 1, 24, 48, and 72 h following removal of the gauze patch. The mean of erythema and edema scores was given according to the Draize test.

The Score of Primary Irritation (SPI) was calculated for each rat using the following formula:

$$\text{SPI for each rat} = \sum \frac{\text{Erythema and Oedema grade at 1, 24, 48 and 72 h}}{\text{Number of observation}} \quad (2)$$

The scores of erythema and edema at 1, 24, 48, and 72 h were summed and divided by the number of observations for the treated sites. The SPI for the control sites was also calculated similarly.

The difference between the summation of SPI scores of six animals from the treated and control sites were calculated and used for the Primary Irritation Index (PII) determination. The PII was calculated as the arithmetical mean of the SPI values of the six rats. The irritation degree was then categorized as negligible, slight, moderate, or severe irritation based on the PII [20].

$$\text{PII} = \frac{\Sigma\text{SPI}(\text{Test}) - \Sigma\text{SPI}(\text{Base})}{\text{Number of animals}} \quad (3)$$

Histological examination

Histological analyses were performed on skin samples from the skin irritation study. The rats were euthanized after 72 h of experiment was completed. The dorsal skin samples were surgically excised and fixed in 10% (v/v) neutral buffered formalin solution at least 24 h before histopathology analysis was performed. The fixed samples were dehydrated through a graded series of ethanol, cleared in two changes of xylene, and infiltrated to four changes of melted paraffin. The skin samples were, then, embedded in melted paraffin and allowed to harden. Thin sections (3–5 μm) were cut using a rotary microtome equipped with disposable steel knives. Sections were flattened on a heated water bath, floated onto microscope slides, and allowed to dry before staining. The sections were, then, stained with hematoxylin and eosin (H&E). The slides were mounted with DPX and covered with a coverslip on top. Slides were examined qualitatively under a light microscope equipped with image-capture facility.

Statistical analysis

All data were presented as a mean±SEM. GraphPad InStat version 3.05 (GraphPad Software, Inc.) was used for statistical analysis. Two-way repeated measure analysis of variance (ANOVA) was used to assess significant differences in the study, followed by a Bonferroni post-hoc multiple comparison. p values of <0.05 (*p<0.05) were considered significant.

RESULTS AND DISCUSSION

RSM-box-behnken optimization of olive oil nanoemulsion formulation

Formulation of nanoemulsions is influenced by a variety of formulation parameters; hence, optimization is essential [21]. The aim optimizing pharmaceutical formulations is generally to determine the levels of the variables, from which a robust product with high quality characteristics may be produced [22]. RSM is the most relevent multivariate statistical techniques that can help to optimize several factors simultaneously. RSM is widely used in the optimization of analytical procedures at present to develop and optimize new formulations due to its advantages over classical one-variable-a-time optimization, such as being able to evaluate all combinations of potential factors at the same time to generate large amounts of information for attaining the best system performance [15]. Moreover, the interacting effect between the variables on the response can be investigated. In addition, this experimental methodology generates a mathematical model that can be visualized in graphical form [17].

The Box-Behnken is a good design for RSM, because it allows estimation of the parameters of the quadratic model and building of sequential designs. The output of this design generates lack of fit to detect the degree of the fitness of the model and uses blocks to run the experiment. The use of Box-Behnken design is slightly more efficient than the central composite design, but it is highly efficient compared to the three-level full factorial designs [23]. In this case, a total of 17 experiments were generated to represent different combinations of factors and their corresponding responses of droplet size and PDI. Regression analysis was carried out to fit mathematical models to the experimental data. A positive value in the regression equation represents an effect that favors optimization due to a synergistic effect, while a negative value indicates an inverse relationship or antagonistic effect between the factor and the response [24]. The approximation of responses was fitted to three mathematical models, linear, interaction, and quadratic. The degree of fitness of three models was evaluated through R^2, p-value, and lack-of-fit. The ANOVA for the response surfaces of quadratic polynomial models advocated the significance of relationships (p<0.05) for all the models. Three dimensional plots were generated to illustrate the response surfaces for the intended models [25].

Fitting the response surface model

The experimental data obtained for every run are shown in Table 1 which shows that the smallest droplet size was 119 nm and the largest was 2541 nm. The minimum and maximum PDI values were in the range of 0.084–0.627, respectively.

The R^2 value for the models of droplet size (0.9833) and PDI (0.9382) suggested a relatively high goodness of fit to predict the true variation of the observed response. The higher p-value (p<0.0001) and lack-of-fit (p=0.6457) for the droplet size model reflect to the significant difference between the independent and dependent variables. Similarly, the PDI model showed a statistical significance of regression (p=0.0018) and non-significant lack-of-fit (p=0.2368). It was observed that independent variables of oil (−213.63) and Span 80 (−850.75) compositions showed negative effects, while Labrasol (259.63) revealed positive effects on the droplet size. Similar observations of the relationship were found for the PDI (Table 2). The quadratic polynomial model was best fitted for the droplet size and PDI between independent and dependent variables.

The model proposed the following polynomial equation for droplet size and PDI:

Final equation in terms of coded factors for response Y_1:

$$\text{Droplet size } (Y_1) = +207.40 - 213.63X_1 - 850.75X_2 + 259.632X_3 + 324.18X_1^2 + 834.93X_2^2 - 3.33X_3^2 - 73.00X_1X_2 - 271.75X_1X_3 - 387.50X_2X_3$$

Table 1: Observed responses for the 17 formulations of Box-Behnken design of olive oil NE

Formulation	Olive oil (w/w, %)	Span 80 (w/w, %)	Labrasol (w/w, %)	Droplet size (nm) (Y_1)	PDI (Y_2)
1	20	5	12.5	2358	0.616
2	70	5	12.5	2173	0.515
3	20	50	12.5	706	0.133
4	70	50	12.5	229	0.289
5	20	27.5	0	311	0.253
6	70	27.5	0	603	0.470
7	20	27.5	25	1269	0.594
8	70	27.5	25	202	0.175
9	45	5	0	1142	0.423
10	45	50	0	312	0.333
11	45	5	25	2541	0.627
12	45	50	25	161	0.286
*13	45	27.5	12.5	530	0.223
*14	45	27.5	12.5	119	0.084
*15	45	27.5	12.5	128	0.097
*16	45	27.5	12.5	331	0.416
*17	45	27.5	12.5	136	0.134

Table 2: Mathematical relationship in the form of factors coefficients and its corresponding p-values for the measured responses of olive oil NE

Regression coefficient	Droplet size (nm)	PDI
Intercept (βo)	207.40	0.12
A (X_1)	−213.63	−0.025
B (X_2)	−850.75	−0.14
C (X_3)	259.63	0.032
A2(X_1^2)	324.18	0.10
B2(X_2^2)	834.93	0.16
C2(X_3^2)	−3.33	0.13
AB (X_1X_2)	−73.00	0.064
AC (X_1X_3)	−271.75	−0.15
BC (X_2X_3)	−387.50	−0.063
R^2	0.9833	0.9382
Regression (*p*-value)	< 0.0001	0.0018
Lack of fit (*p*-value)	0.6457	0.2368

A: olive oil; B: Span 80; C: Labrasol

Final equation in terms of coded factors for response Y_2:

$$PDI\ (Y_2) = +0.12 - 0.025X_1 - 0.14X_2 + 0.032X_3 + 0.10X_1^2 + 0.16X_2^2 + 0.13X_3^2 + 0.064X_1X_2 - 0.15X_1X_3 - 0.063X_2X_3$$

As there was a significant relationship between the independent variables for the measured responses (droplet size and PDI), the quadratic polynomial model of droplet size and PDI were statistically analyzed using ANOVA (Table 3). In the case of droplet size, A, B, C, AB, AC, B^2, and C^2 showed significant model terms ($p<0.05$), whereas for PDI, the B, AB, AC, BC, and B^2 are significant model terms ($p<0.05$). Although all factors offered significant effects, Span 80 seems to be the most influential factor that determined the droplet size as it has higher significant linear and interaction effects($p<0.0001$).

The analyses of response surface were plotted in three-dimensional model graphs. As shown in Fig. 1, the quadratic terms of all independent variables have significant effects on the droplet size. An inverse relationship of the composition of oil and Span 80, while a direct proportional relationship of the Labrasol on the droplet size, implied that increasing the composition of oil and surfactant while decreasing the cosurfactant caused a reduction in the droplet size. By keeping constant the two factors, a lower composition of Span 80 at 5% produced a larger droplet size. Increasing the Span 80 concentration to 27.5% reduced the droplet size, but additional increases above 27.5% of Span 80 led to an increase of droplet size again.

Droplet size is a key characteristic of nanoemulsion stability, contributing greatly to the physical stability and organoleptic properties

Table 3: Analysis of variance of the response surfaces of the fitted quadratic equation for droplet size and PDI of olive oil NE

Parameter	Droplet size		PDI	
Main (Linear) effect	**f-value**	**p-value**	**F-value**	**p-value**
A (X_1)	13.50	0.0079	0.98	0.3541
B (X_2)	214.07	<0.0001	31.67	0.0008
C (X_3)	19.94	0.0029	1.61	0.2451
Interaction effect				
AB (X_1X_2)	16.36	0.0049	8.70	0.0214
AC (X_1X_3)	108.51	<0.0001	21.20	0.0025
BC (X_2X_3)	0.001721	0.9681	14.29	0.0069
Quadratic effect				
A^2(X_1^2)	0.79	0.4042	3.22	0.1159
B^2(X_2^2)	10.92	0.0130	16.51	0.0048
C^2(X_3^2)	22.21	0.0022	3.07	0.1232

A: Olive oil; B: Span 80; C: Labrasol

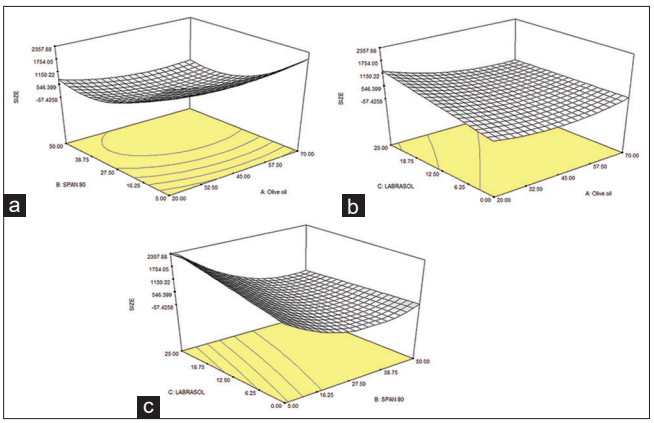

Fig. 1: The effect of Span 80 and olive oil on droplet size (a), the effect of Labrasol and olive oil on droplet size (b) and the effect of Labrasol, and Span 80 on droplet size (c)

of nanoemulsion, where large globules tend to coalesce faster than the small ones as in Ostwald ripening. Thus, in theory, nanoemulsion stability can be achieved by reducing the size of the droplets [26]. Apparently, at low concentrations of Span 80, this surfactant did not completely adsorb to the surface of freshly formed droplets; thus, they coalescence, leading to an increase in the droplet size. Similar results have been reported for the optimum use of emulsifiers and droplet size [21,27]. Increasing the Span 80 concentration would result in a greater number of available surfactant to lower the interfacial tension

and sufficiently cover the surface area of droplet population, thus, decreasing the overall droplet size [17,25]. Unexpectedly, an additional increase in Span 80 composition (27.5–50%) resulted in an increase of the droplet size. Further increase in the surfactant concentration after the surfactant reaches its saturation adsorption plateau caused a larger droplet size due to the reduction in the surface excess. The surface excess is defined as the number of moles of surfactant adsorbed per unit area of the interface [25]. The previous studies reported that excessive amounts of surfactant affect the diffusion rate of surfactant as well as the adsorption of surfactant-droplet and, therefore, leads to an increase in the coalescence of the droplets [28-30]. Another explanation for the larger droplet size is associated with the depletion-flocculation mechanism of adsorbed surfactant. Increasing the surfactant concentration above its critical point causes molecules of surfactant to form micelles in the continuous phase rather than oriented on the particle surface, resulting in the increasing of the local osmotic pressure. Hence, the continuous phase between some droplets moves to them, which causes the depletion of the continuous phase between the drops. Consequently, aggregation takes place and the particle size increases [27].

PDI characterizes the disperse systems with respect to deviation from the average size and can vary from 0 to 1 with PDI = 0.250 being an acceptable value for emulsion [17]. In case of PDI, only the quadratic terms of Span 80 had a significant negative effect on the PDI value with p-value of 0.0008 (Fig. 2). Since the responses were affected by the interaction of independent variables, presenting a quadratic relationship suggested a non-linear relationship between factors and responses.

Verification of the model

After analyzing the effects of the independent variables on the responses, the optimized formula was determined (Table 4). In an optimum nanoemulsion, the droplet size is in the range of 119–500 nm and the PDI value is within 0.084–0.400.

Experimental and predicted values of the responses were compared to check the adequacy of the response surface equations. The numerical optimization method was employed for optimization of the composition of nanoemulsion formulations. The experimental outcomes were obtained by preparing the nanoemulsion using the optimized formula as suggested by the software. It was observed that the experimental outcomes were not significantly different with the predicted outcomes suggesting a high fit of the polynomial response surface (Table 5).

Droplet size and PDI

The average droplet size of the olive oil NE was 144.2±0.8 nm. PDI characterizes the disperse systems with respect to the deviation from the average size exhibited. It was found that the PDI value of olive oil NE was 0.105±0.014. The information regarding droplet size and size distribution is very important for the characteristic of nanoemulsion since its determines the rate and extent of drug release as well as absorption. The smaller the droplet size, the larger the interfacial surface area will be available for drug absorption [31]. A small droplet size is the primary determinant for oil droplets to fuse with the skin in providing a channel for drug delivery [32]. In general, it was suggested that the droplet sizes below 200 nm are sufficient for delivery to deeper layers [33]. In this study, the percentage oils used was 15.9%. The mean droplet size reported in the literature ranged from about 200 to 300 nm for 10 % and 20 % of oil used in the nanoemulsion. Therefore, the size obtained from this study was in line with the previous studies.

pH

The pH of the topical formulation is very important as topical products that are too acidic or alkaline may cause skin irritation that can lead to bacterial infection. Therefore, an ideal topical product should have a pH that is close to the pH of a healthy human skin (4.5–5.5) [34]. It has been observed that the pH of the formulation was 6.82±0.01 which is close to the pH of the skin, thus suitable to be used without affecting the natural pH of the human skin.

Transmission electron microscopy

The electron micrographs showed that the oil droplets were brighter and more circular in shape and dispersed in the water phase (Fig. 3).

Skin irritation studies

Assessment of skin irritation topical products is necessary to allow biologic evaluation of products biocompatibility. This assessment can be done by means of *in vivo* and *in vitro* tests to determine the risk of irritation due to the contact between the products and human skin. The

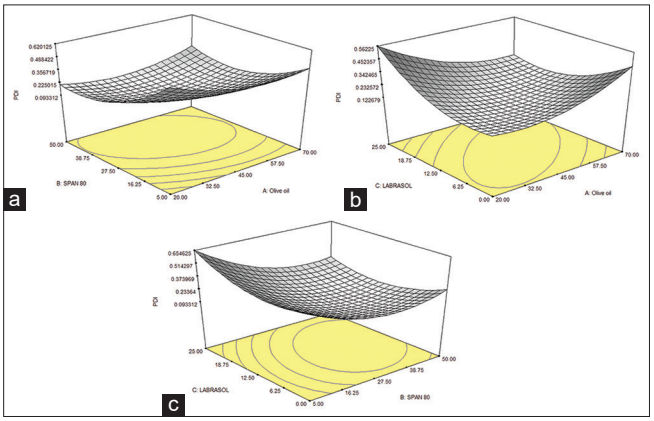

Fig. 2: The effect of Span 80 and olive oil on PDI (a), the effect of Labrasol and olive oil on PDI (b) and the effect of Labrasol, and Span 80 on PDI (c)

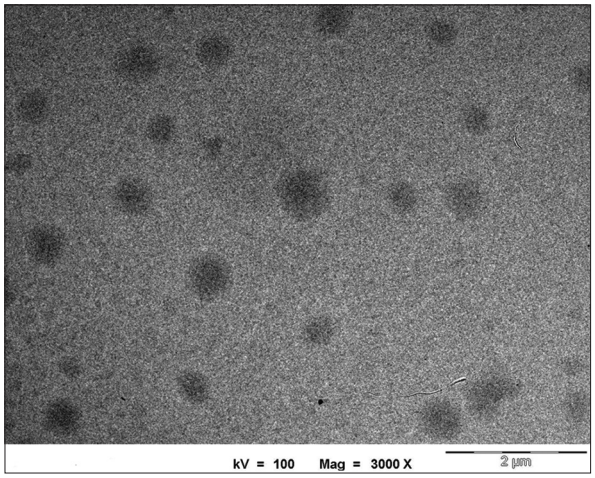

Fig. 3: TEM image of olive oil NE (3000× magnification)

Table 4: Numerical optimization specification for the define droplet sizes and PDI of olive oil NE.

Parameter	Goal	Low level	High level
Olive oil	is in range	20	70
Span 80	is in range	5	50
Labrasol	is in range	0	25
Droplet sizes (nm)	is in range	119	500
PDI	is in range	0.084	0.400

Table 5: The predicted and observed response values for the optimized olive oil NE

Response	Droplet size (nm)	PDI
Predicted	140.0	0.099
Observed	144.2	0.105

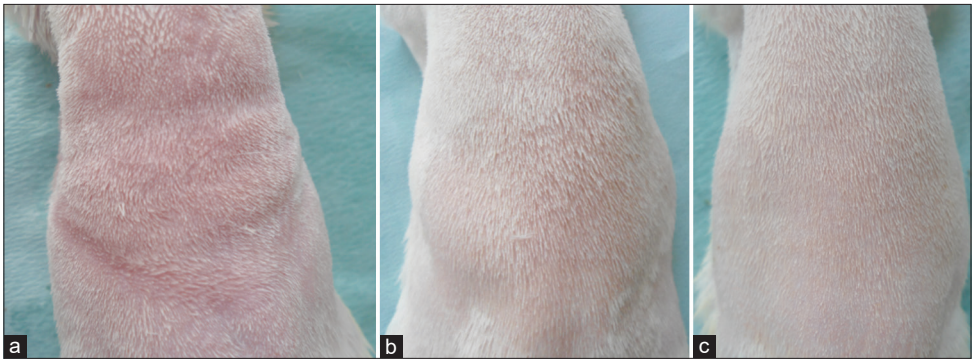

Fig. 4: Gross appearance skin irritancy after 24 h. 20% SLS as positive control (a), normal saline as negative control (b), and olive oil NE (c)

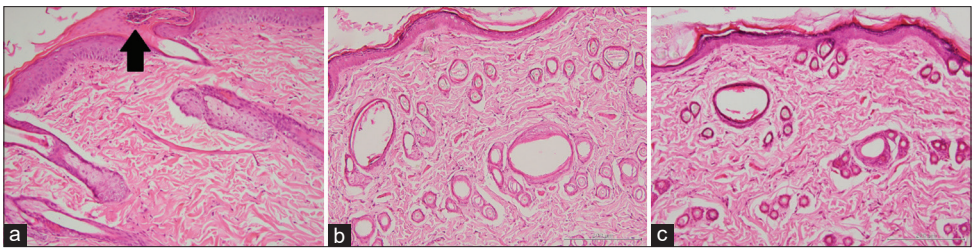

Fig. 5: Photomicrographs are showing the histology of the skin irritancy of rats after 72 h of treatment (×20 magnification and scale bar 200 μm). Standard irritant showed mild parakeratosis (nuclei at the stratum corneum) at the epidermis layer of the skin as indicated by the arrow. 20% SLS as positive control (a), normal saline as negative control (b), and olive oil NE (c)

skin irritation test was followed according to the OECD test guideline 404. In animal tests, the test substances are to be applied on the animal's shaved skin and the results from the Draize skin irritancy test will be scored based on physiological observations on the animals [35]. Strong irritant substances cause erythema and edema and a variety of histological changes such as erosion or ulcer and inflammation that ranges in severity depending on the intensity. Mild or moderate amounts of the irritant substances on the other hand, does not erode the epidermis, but it shows reactive changes such as hyperkeratosis and acanthosis [36]. Absence of these reactions reflects no irritancy. Fig. 4 depicts the visual observation of rat's skin irritation after 24 h of application. Rats treated with topical application of olive oil NE did not demonstrate any skin reactions of irritation such as erythema (redness) and edema (swelling). Only the standard irritant positive control group that received 20 % (w/v) SLS triggered itching, resulting in visible skin irritation as shown by slight reddening of the skin that was observed within 24 h after application.

Histological examination

Histological examination of rat skin samples stained with H&E after 72 h of experiment is shown in Fig. 5. Pathological features of irritation such as parakeratosis, spongiosis, or acanthosis were not observed in rats receiving olive oil NE. In contrast, the standard irritant group showed mild parakeratosis (nuclei at the stratum corneum) at the epidermis layer of the skin as indicated by the arrow with no neutrophilic or lymphocytic infiltration in the dermis reflecting to slight irritation. The Draize test PII values range from 0 to 8, corresponding to no irritation to severe irritation. The PII score for olive oil NE was zero, whereas the standard irritant group was calculated to be 1.3 PII, reflecting to the slight irritation in this group. The data from visual observation, histological study, and PII calculation were correlated well. Hence, it can be concluded that olive oil NE was non-irritating to the skin. Thus, the developed formulation is considered safe to be applied for topical drug delivery.

CONCLUSION

In this study, the therapeutic potential of olive oil in a nanoemulsion has been successfully developed for a topical application. The development and optimization of the nanoemulsion were carried out using RSM to select the best levels of parameters in producing robust and high-quality formulations. The composition of oil, surfactant, and cosurfactant was the parameters evaluated and the nanoemulsion's small droplet size with good PDI as the responses. The optimized olive oil NE was found to have the droplet size of 144 nm with the PDI value of 0.104. Assessment of skin irritancy potential of olive oil NE demonstrated that the formulation is not a skin irritant; therefore, it is acceptable for topical application.

ACKNOWLEDGEMENTS

This study was funded by Universiti Kebangsaan Malaysia under the grant number ERGS/1/2013/SKK02/UKM/02/3. Additional financial support for the scholar, Wan Maznah Binti Wan Ishak was received from Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang.

AUTHOR CONTRIBUTIONS

WMWI performed the experiments, collected, and analysed the data as well as wrote the manuscript.

MHZ designed the entire study, provided supervision, and contributed to the manuscript revision.

WMWI and MHZ read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

REFERENCES

1. Tavakoli S, Klar AS. Advanced hydrogels as wound dressings. Biomolecules 2020;10:1169. doi: 10.3390/biom10081169, PMID 32796593
2. Silna EA, Krishnakumar K, Nair SK, Narayanan AV. Hydrogels in topical drug delivery-a review. Int J Innov Drug Discov 2016;6:87-93.
3. Bouchemal K, Briançon S, Perrier E, Fessi H. Nano-emulsion formulation using spontaneous emulsification: Solvent, oil and

surfactant optimisation. Int J Pharm 2004;280:241-51. doi: 10.1016/j.ijpharm.2004.05.016, PMID 15265563
4. Abolmaali SS, Tamaddon AM, Farvadi FS, Daneshamuz S, Moghimi H. Pharmaceutical nanoemulsions and their potential topical and transdermal applications. Iran J Pharm Sci 2011;7:12.
5. Al-Edresi S, Baie S. *In-vitro* and *in-vivo* evaluation of a photo-protective kojic dipalmitate loaded into nano-creams. Asian J Pharm Sci 2010;16:251-65.
6. Rabasco Alvarez AM, González Rodríguez ML. Lipids in pharmaceutical and cosmetic preparations. Grasas Aceites 2000;51:74-96. doi: 10.3989/gya.2000.v51.i1-2.409
7. Taguchi K, Fukushima S, Yamaoka Y, Takeuchi Y, Suzuki M. Enhancement of propylene glycol distribution in the skin by high purity cis-unsaturated fatty acids with different alkyl chain lengths having different double bond position. Biol Pharm Bull 1999;22:407-11. doi: 10.1248/bpb.22.407, PMID 10328563
8. Schürer N, Schliep V, Williams ML. Differential utilization of linoleic and arachidonic acid by cultured human keratinocytes. Skin Pharmacol 1995;8:30-40. doi: 10.1159/000211328, PMID 7786523
9. Cicerale S, Lucas L, Keast R. Biological activities of phenolic compounds present in virgin olive oil. Int J Mol Sci 2010;11:458-79. doi: 10.3390/ijms11020458, PMID 20386648
10. Ichihashi M, Ahmed NU, Budiyanto A, Wu A, Bito T, Ueda M, *et al*. Preventive effect of antioxidant on ultraviolet-induced skin cancer in mice. J Dermatol Sci 2000;23:S45-50. doi: 10.1016/s0923-1811(00)00083-9, PMID 10764992
11. Cardoso CR, Souza MA, Ferro EA, Favoreto S, Pena JD. Influence of topical administration of n-3 and n-6 essential and n-9 nonessential fatty acids on the healing of cutaneous wounds. Wound Repair Regen 2004;12:235-43. doi: 10.1111/j.1067-1927.2004.012216.x, PMID 15086775
12. Bhatt P, Madhav S. A detailed review on nanoemulsion drug delivery system. Int J Pharm Sci Res 2011;2:2482-9.
13. McClements DJ. Nanoemulsions versus microemulsions: Terminology, differences, and similarities. Soft Matter 2012;8:1719-29. doi: 10.1039/C2SM06903B
14. Wani TA, Ahmad A, Zargar S, Khalil NY, Darwish IA. Use of response surface methodology for development of new microwell-based spectrophotometric method for determination of atrovastatin calcium in tablets. Chem Cent J 2012;6:134. doi: 10.1186/1752-153X-6-134, PMID 23146143
15. Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 2008;76:965-77. doi: 10.1016/j.talanta.2008.05.019, PMID 18761143
16. Gunst RF. Response surface methodology: Process and product optimization using designed experiments. Technometrics 1996;38:284-6. doi: 10.1080/00401706.1996.10484509
17. Zainol S, Basri M, Basri HB, Shamsuddin AF, Abdul-Gani SS, Karjiban RA, *et al*. Formulation optimization of a palm-based nanoemulsion system containing levodopa. Int J Mol Sci 2012;13:13049-64. doi: 10.3390/ijms131013049, PMID 23202937
18. Singh G, Pai RS, Devi VK. Response surface methodology and process optimization of sustained release pellets using Taguchi orthogonal array design and central composite design. J Adv Pharm Technol Res 2012;3:30-40. doi: 10.4103/2231-4040.93565, PMID 22470891
19. Aqil M, Kamran M, Ahad A, Imam SS. Development of clove oil based nanoemulsion of olmesartan for transdermal delivery: Box-behnken design optimization and pharmacokinetic evaluation. J Mol Liq 2016;214:238-48. doi: 10.1016/j.molliq.2015.12.077
20. More BH, Sakharwade SN, Tembhurne SV, Sakarkar DM. Evaluation for skin irritancy testing of developed formulations containing extract of *Butea monosperma* for its topical application. Int J Toxicol Appl Pharmacol 2013;3:10-3.
21. Hosseini S, Tarzi BG, Gharachorloo M, Ghavami M, Bakhoda H. Optimization on the stability of linseed oil-in-water nanoemulsions generated by ultrasonic emulsification using response surface methodology (RSM). Orient J Chem 2015;31:1223-30. doi: 10.13005/ojc/310282
22. Villar AM, Naveros BC, Campmany AC, Trenchs MA, Rocabert CB, Bellowa LH. Design and optimization of self-nanoemulsifying drug delivery systems (SNEDDS) for enhanced dissolution of gemfibrozil. Int J Pharm 2012;431:161-75. doi: 10.1016/j.ijpharm.2012.04.001, PMID 22498011
23. Ferreira SL, Bruns RE, Ferreira HS, Matos GD, David JM, Brandão GC, *et al*. Box-behnken design: An alternative for the optimization of analytical methods. Anal Chim Acta 2007;597:179-86. doi: 10.1016/j.aca.2007.07.011, PMID 17683728
24. Kotta S, Khan AW, Ansari SH, Sharma RK, Ali J. Formulation of nanoemulsion: A comparison between phase inversion composition method and high-pressure homogenization method. Drug Deliv 2013;22:455-66.
25. Alzorqi I, Ketabchi MR, Sudheer S, Manickam S. Optimization of ultrasound induced emulsification on the formulation of palm-olein based nanoemulsions for the incorporation of antioxidant β-D-Glucan polysaccharides. Ultrason Sonochem 2016;31:71-84. doi: 10.1016/j.ultsonch.2015.12.004, PMID 26964925
26. Barry-Ryan C, Gaston E, Frias JM, Burke R, Traynor M. Formation and stability of an oil in water emulsion containing lecithin, xanthan gum and sunflower oil. Int Food Res J. 2013;20(5):2173-81.
27. Hasani F, Pezeshki A, Hamishehkar H. Effect of surfactant and oil type on size droplets of betacarotene bearing nanoemulsions. Int J Curr Microbiol Appl Sci 2015;4:146-55.
28. Li PH, Chiang BH. Process optimization and stability of D-limonene-in-water nanoemulsions prepared by ultrasonic emulsification using response surface methodology. Ultrason Sonochem 2012;19:192-7. doi: 10.1016/j.ultsonch.2011.05.017, PMID 21680223
29. Chanana GD, Sheth BB. Particle size reduction of emulsions by formulation design-II: Effect of oil and surfactant concentration. PDA J Pharm Sci Technol 1995;49:71-6. PMID 7780748
30. Esquena J, Forgiarini A, González C, Solans C. Formation and stability of nanoemulsions in mixed nonionic surfactant systems. Prog Colloid Polym Sci 2001;118:184-9.
31. Shafiq S, Shakeel F, Talegaonkar S, Ahmad FJ, Khar RK, Ali M. Development and bioavailability assessment of ramipril nanoemulsion formulation. Eur J Pharm Biopharm 2007;66:227-43. doi: 10.1016/j.ejpb.2006.10.014, PMID 17127045
32. Modi JD, Patel JK. Nanoemulsion-based gel formulation of aceclofenac for topical delivery. Int J Pharm Pharm Sci Res 2011;1:6-12.
33. Çelik B, Sağıroğlu AA, Özdemir S. Design, optimization and characterization of coenzyme Q10-and D-Panthenyl triacetate-loaded liposomes. Int J Nanomedicine 2017;12:4869-78. doi: 10.2147/IJN.S140835, PMID 28744121
34. Mahdi ES, Noor AM, Sakeena MH, Abdullah GZ, Abdulkarim MF, Sattar MA. Formulation and *in vitro* release evaluation of newly synthesized palm kernel oil esters-based nanoemulsion delivery system for 30 % ethanolic dried extract derived from local phyllanthus urinaria for skin antiagin. Int J Nanomedicine 2011;6:2499-512. doi: 10.2147/IJN.S22337, PMID 22072884
35. James O, Sunday AB. Evaluation of acute dermal irritation and wound contraction by *Gymnema sylvestre* and *Datura Metel* extracts in rats. Am J Biomed Life Sci 2014;2:83-8. doi: 10.11648/j.ajbls.20140204.14
36. Mitsuishi M, Oshikata T, Kumabe S, Kobayashi A, Katoku K, Kanno T, *et al*. Histological dermal changes caused by preparation and application procedures in percutaneous dose toxicity studies in dogs, rabbits and rats. J Toxicol Pathol 2015;28:1-9. doi: 10.1293/tox.2014-0021, PMID 26023255