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Research Article

Optimization of celecoxib nanoemulsion formulated using nutmeg oil as a carrier oil by central composite design: *In-vitro* and *in-vivo* evaluation

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ABSTRACT

Celecoxib (CLX) is a selective COX-2 inhibitor used in the management of osteoarthritis and rheumatoid arthritis, but its long-term use is associated with gastrointestinal, renal, and cardiovascular side effects. This study aimed to optimize a nanoemulsion (NE) formulation of CLX using nutmeg oil (NMO) as a carrier, and Tween 80–PEG 400 as surfactant and co-surfactant, respectively, to enhance transdermal delivery. A Central Composite Design (CCD) was employed to optimize the effects of formulation variables on droplet size, zeta potential, transmittance, and polydispersity index. The optimized formulation (NECLX13) demonstrated a droplet size of 48.30 ± 1.30 nm, zeta potential of -29.30 ± 2.41 mV, transmittance of 95.22 ± 1.15%, and a polydispersity index of 0.488 ± 0.015. In vitro skin permeation studies using Franz diffusion cells and shed snake skin showed significantly enhanced drug flux compared to individual CLX in surfactant, co-surfactant, or NMO alone. NECLX13 reduced carrageenan-induced paw edema in rats within 30 minutes based on the *in vivo* test and maintained anti-inflammatory effects for up to 6 hours, comparable to Voltaren Emulgel®. These findings indicate that the optimized nanoemulsion enhances the topical delivery of CLX and could be a promising alternative for the treatment of inflammatory conditions.

Keywords:
Celecoxib; Central composite design; Nanoemulsion; Nutmeg oil; Anti-inflammation; Transdermal delivery

1. INTRODUCTION

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) is currently the first line of osteoarthritis (OA) therapy in Indonesia[1]. This therapy can cause gastric ulcers in OA patients, especially in geriatrics[2]. There is a significant relationship between the occurrence of gastric ulcers in geriatrics who are given NSAIDs[3,4]. One of NSAIDs with low gastric side effects is celecoxib (CLX). Chan et al. stated that neither CLX nor NSAID effectively prevents the recurrence of ulcers[5]. The limitation of CLX is the low solubility and bioavailability. The surface area of the active substance must be increased in order to extend the effect of the active substance[6]. Many techniques that can improve solubility including nano-sized delivery[7].

Nanoemulsion are dispersions of immiscible liquid phase with droplet size below 200 nm. Nanoemulsion is considered an advanced nano-droplet system for systemic, controlled, and targeted drug

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Table 1. Upper and lower limits of the formulation factor of nutmeg oil-based celecoxib nanoemulsion

Factor	Factor Level (		Targeted Response			
	-1	+1	Droplet Size (Y1)	Zeta Potential (Y2)	PI (Y3)	Transmittance Percentage (Y4)
NMO (A)	3	10	50-200 nm	-30-50 mV	0.2-0.8	85-100%
Tween 80 (B)	20	40				
PEG 400 (C)	25	55				

Dependent variables: Y1: droplet size; Y2: zeta potential; Y3: poydispersity index; Y4: transmittance percentage

delivery especially for topical delivery[8]. This administration route bypasses the hepatic metabolism to reach systemic circulation. The critical parameters in nanoemulsion formulation is the composition of surfactant-co-surfactant-oil[9]. Surfactants not only enhance skin permeability by disrupting the *stratum corneum* structure but also play a vital role in reducing interfacial tension, stabilizing the emulsion system, and modulating droplet size[10]. They contribute to improved drug solubilization and bioavailability[11]. Co-surfactants assist by further lowering interfacial tension and reducing bending stress, while carrier oils aid in increasing the solubility of the active drug component[12]. The type and concentration of surfactant significantly influence the final droplet size of the nanoemulsion. Smaller droplet sizes provide a larger surface area, which can improve drug absorption and bioavailability. Carrier oil can help increasing the drug's solubility[13]. Some carrier oils for example nutmeg oil, avocado oil, coconut oil have inherent therapeutic properties such as anti-inflammatory, antimicrobial, or soothing effects which can synergize with the active drug to enhance efficacy[14].

There were several CLX nanoemulsion researches yet they were for oral preparation or did not use natural plant oil as the carrier[15–18]. Nanoemulsion based on natural oils have been investigated for a number of uses, including aromatherapy, antimicrobial, and anti-inflammatory[19]. An Indonesian native plant which produces an essential oil is nutmeg seed. This essential oil potentially can be a pain chronic reliever[20]. Nutmeg oil (NMO) has been shown not to irritate the skin and can penetrate the *stratum corneum* layer to increase the absorption of active substances transdermal[21]. NMO contains of myristicin and β-phellandrene which traditionally used for inflammation and reducing stress muscle[22].

The objective of this study was to determine the optimum formulation of a CLX nanoemulsion using Central Composite Design (CCD) (write the design value used) by varying surfactant, co-surfactant, and NMO concentrations to enhance the drug's permeation through the skin and effectivity. This study addresses that gap by developing a CCD-optimized nanoemulsion formulation using nutmeg oil, targeting both improved permeation and synergistic anti-inflammatory effects. Additionally, CCD offers a significant advantage by providing highly accurate predictive outcomes.

2. MATERIALS AND METHODS

2.1. Materials

Celecoxib (99%) was obtained from Gz Tangrong Tech. Co. Ltd Hong Kong, Republic of China (No. 1272/2008 EU-GHS/CLP) with pharmaceutical grade, nutmeg oil obtained from PT Daarjeling Aroma, Bandung, Indonesia with cosmetics grade. Tween 80 and PEG 80 obtained from Brataco, Semarang, Indonesia with pharmaceutical grade, benzyl alcohol obtained from PT Multi Kimia Raya, Semarang, Indonesia with pharmaceutical grade. Other materials for analysis such as ethanol 96%, KH_2PO_4 and Na_2HPO_4 were obtained from Merck® with analytical grade. Voltaren Emulgel® was obtained from Wahid Hasyim Pharmacy, Semarang, Indonesia. Shed snake skin (*Python morulus*) was obtained from Semarang Zoo, Semarang City, Indonesia.

2.2. Optimization of CLX nanoemulsion

This study employed a purely experimental approach using the Central Composite Design (CCD) in Design Expert® StateEase version 13.0.5 to optimize three factors: the concentrations of NMO, Tween 80, and PEG 400. The CCD comprised 8 factorial points (2^3) a low and high levels with 6 axial points (2 per factor) and 6 center points. The value of α (alpha) used was 1.68179, selected to ensure rotatability of the design. This places the axial points at a distance of ±1.68179 from the center point in each variable dimension.

Upper and lower limits of the formulation factor of nutmeg oil-based CLX nanoemulsion can be seen in Table 1. The prediction of CLX nanoemulsion with variation of NMO as a carrier, Tween 80 as surfactant and PEG 400 as co-surfactant is presented in Table 2.

2.3. Preparation of CLX nanoemulsion

CLX nanoemulsion was prepared using high speed homogenizer (Ultra Thurrax® T 18 mini digital, Wahid Hasyim University, Semarang, Indonesia)[23]. Briefly, CLX is dissolved in PEG with a magnetic stirrer at a speed of 700 rpm for 10 minutes, then NMO was

Table 2. The prediction of CLX nanoemulsion with variation of nutmeg oil as a carrier, tween 80 as surfactant and PEG 400 as co-surfactant

Run	Factor 1	Factor 2	Factor 3
	A: Nutmeg Oil (%)	B: Tween 80 (%)	C: PEG 400 (%)
1	3	40	55
2	3	40	25
3	6.5	30	40
4	10	20	55
5	6.5	30	40
6	3	20	55
7	6.5	30	14.7731
8	6.5	13.1821	40
9	10	40	25
10	6.5	30	40
11	3	20	25
12	6.5	46.8179	40
13	6.5	30	40
14	6.5	30	40
15	12.3863	30	40
16	6.5	30	65.2269
17	0.613725	30	40
18	6.5	30	40
19	10	40	55
20	10	20	25

added to the mixture. The mixing process continued at the same speed at a temperature of 35°C (mixture A). Tween 80 were heated at 35°C (mixture B). Mixture A was poured into mixture B and homogenized using high speed homogenizer Ultra Thurrax® at a speed of 3000 rpm for 10 minutes at 30°C. The mixing was continued for 10 minutes or until the clear and transparent nanoemulsion was formed. The manufacturing method was according to the previous research with a slight modification[24].

2.4. Characterization of CLX nanoemulsion

2.4.1. Organoleptic test

The optimized formula was evaluated for its color, homogeneity, and phase separation. The test was conducted on the CLX optimum formula based on the CCD.

2.4.2. Particle size analysis

A 0.2 mL of formulation was diluted with 1.8 mL of deionized water in disposable cuvettes and analyzed using a particle size analyzer (Horiba SZ-100, Indonesia Islamic University, Yogyakarta, Indonesia) with dynamic light scattering (DLS) method with the frequency of 0 to 7%.

2.4.3. pH

The pH of the optimum formula (NECLX13) was determined by using a digital pH meter. A 1 gm of each formulation was dispersed in 10 gm of purified water separately. All the dispersions were shaken properly and determined triplicate the pH using digital pH meter (Electrolab®) in Wahid Hasyim University, Semarang, Indonesia.

2.4.4. Viscosity

Viscosity of NECLX13 was determined using a viscometer at 100 rpm for 1 min with cone spindle number 04. The test was run triplicate using cone and plate viscometer (Rheosys Merlyn II®) in Ahmad Dahlan University Laboratory, Yogyakarta, Indonesia.

2.4.5. Microscopy images test

The optimized formula (NECLX13) sample was dropped on the grid, absorbed and dried. Then, the staining agent uranyl acetate solution was dropped, absorbed and dried, then measured with Transmission Electron Microscope (TEM) at 100 kV. The instrument used was JEOL JEM-1400® in Chemical Laboratory of Gadjah Mada University, Yogyakarta, Indonesia[25].

2.5. *In vitro* permeation test of optimum CLX nanoemulsion

A Franz-Diffusion cell with a shed snake skin (*Python morulus*) was employed to conduct the *in vitro* drug release study of the optimum formula of CLX-NE (Group I). The dorsal section of the snake skin was rinsed with distilled water and immersed in distilled water for 24 hours. The membrane was elevated and air-dried at ambient temperature by positioning it on filter paper to expedite the drying process. This shed skin with a diameter of 2.5 cm, prepared for utilization[26,27]. The test was run with continuous agitation

Table 3. The statistical model of droplet size, zeta-potential, polydispersity index and transmittance percentage

Responses	Model Sig.	Lack-of-fit	R^2	Adjusted R^2
Droplet Size (nm)	0.00001	0.0532	0.9891	0.9792
Zeta Potential (mV)	0.0018	0.1665	0.8793	0.7605
Polydispersity Index	0.0019	0.4531	0.8728	0.7584
Transmittance Percentage (%)	0.0002	0.9254	0.9182	0.8446

at 50 rpm and a temperature of 37±0.5°C in the acceptor compartment. The membrane surface of the donor compartment was coated with 1 g of the nanoemulsion. The acceptor phase was composed of 200.0 mL of 0.01 M phosphate buffer pH 7.4±0.2 to achieve sink conditions. The acceptor phase (3.0 mL) was sampled at time intervals of 0.5, 1, 2, 3, 4, 5, and 6 hours[28,29]. The amount of CLX released was measured with spectrophotometry in a Shimadzu UV-VIS® (Wahid Hasyim University, Semarang, Indonesia) at 252 nm. In order to facilitate comparison, the same quantity of CLX was dissolved in each carrier or solvent, including NMO, Tween 80, and PEG 400.

2.6. *In vivo* permeation test of optimum CLX nanoemulsion

Ethical approval for the study was granted from Ministry of Health, Semarang Health Polytechnic (Indonesia), with the ethical approval no: 1368/EA/F.XXIII.38/2024. The animal used in this study was rats with species of *Rattus novergicus* age 2-3 months old with weight of 150-200 grams. The animals were obtained from Faculty of Veterinary Gadjah Mada University. The animals were acclimatized at room temperature for 7 days before the induction. The animal was divided into seven groups and induced by 1% carrageenan solution. The groups were categorized as CLX+NMO; NECLX13 (optimum formula); CLX+PEG400, CLX+Tween 80 and control including nanoemulsion base (Tween 80+PEG 400) as a base control; untreated rats as negative control and Voltaren Emulgel® as a positive control. The edema was measured before and thirty minutes after induction and every thirty minutes up to 6 hours. The data obtained were edema volume (mL) and calculated as percentage of inhibition (%)[30].

2.7. Statistical Analysis

The optimization process was analyzed using CCD based on the significance and lack-of-fit value of the model. All of the organoleptic, *in vitro* and *in vivo* permeation data were tested in triplicate (n=3). The *in vitro* and *in vivo* permeation study of CLX nanoemulsion were analyzed statistically using one-way ANOVA with a confidence level of 95%.

Table 4. The result of the observed and predicted value of particle size (Y1), zeta potential (Y2), polydispersity index (Y3) and transmittance percentage (Y4)

Sample	Particle Size (Y1)		Zeta Potential (Y2)		Polydispersity Index (Y3)		Transmittance Percentage (Y4)	
	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted
NECLX1	122.7±1.42	125	-28.5±1.22	-29.7	0.477±0.01	0.476	78.3±2.24	76.5
NECLX2	765.3±2.15	761.5	-33.1±1.17	-32.5	0.748±0.05	0.744	55.1±2.16	54.5
NECLX3	58.8±1.52	57.5	-28.7±1.15	-28.3	0.431±0.02	0.441	79.1±1.14	78.6
NECLX4	1140.5±2.17	1135	-72.5±2.24	-70.3	0.805±0.03	0.784	40.5±1.26	38.1
NECLX5	90.2±2.21	88	-38.1±2.71	-35.5	0.558±0.02	0.541	78.3±1.17	77.2
NECLX6	325.7±1.82	322.5	-33.5±2.65	-32.1	0.503±0.02	0.483	79.5±2.15	78.7
NECLX7	770.6±4.22	776.3	-72.5±2.33	-71.5	0.778±0.04	0.765	42.3±1.28	41.5
NECLX8	785.4±4.15	780.3	-71.2±2.14	-70.5	0.921±0.06	0.875	45.2±1.35	43.2
NECLX9	660.5±4.13	667	-68.5±1.18	-68.1	0.875±0.05	0.863	55.1±2.17	54.3
NECLX10	98.9±2.81	92	-30.1±1.23	-28.4	0.352±0.03	0.341	97.8±1.42	96.3
NECLX11	351.5±3.27	348.5	-55.2±2.27	-52.3	0.678±0.01	0.661	78.5±2.45	77.5
NECLX12	655.6±3.18	653.5	-73.4±2.41	-70.5	0.872±0.02	0.868	55.8±2.71	56.3
NECLX13	45.7±1.22	43.5	-31.3±1.28	-29.3	0.395±0.02	0.381	98.9±1.15	97.6
NECLX14	19.2±3.14	15.5	-30.5±1.57	-28.5	0.365±0.01	0.361	98.1±2.83	97.8
NECLX15	675.8±5.21	678	-70.7±1.41	-71.5	0.864±0.05	0.862	77.5±2.18	78
NECLX16	632.4±4.35	625.5	-71.3±1.38	-70.2	0.871±0.06	0.861	55.2±1.65	54.3
NECLX17	20.1±5.15	15	-28.6±2.19	-30.2	0.515±0.05	0.481	98.1±1.45	97.3
NECLX18	59.6±4.33	58	-12.4±2.23	-12.2	0.218±0.03	0.221	99.2±1.77	98.5
NECLX19	484.5±2.18	481.7	-64.1±1.44	-65.3	0.858±0.05	0.861	77.2±2.86	78.5
NECLX20	590.2±2.12	588.3	-72.1±2.48	-71.2	0.892±0.04	0.881	54.5±2.19	54.3

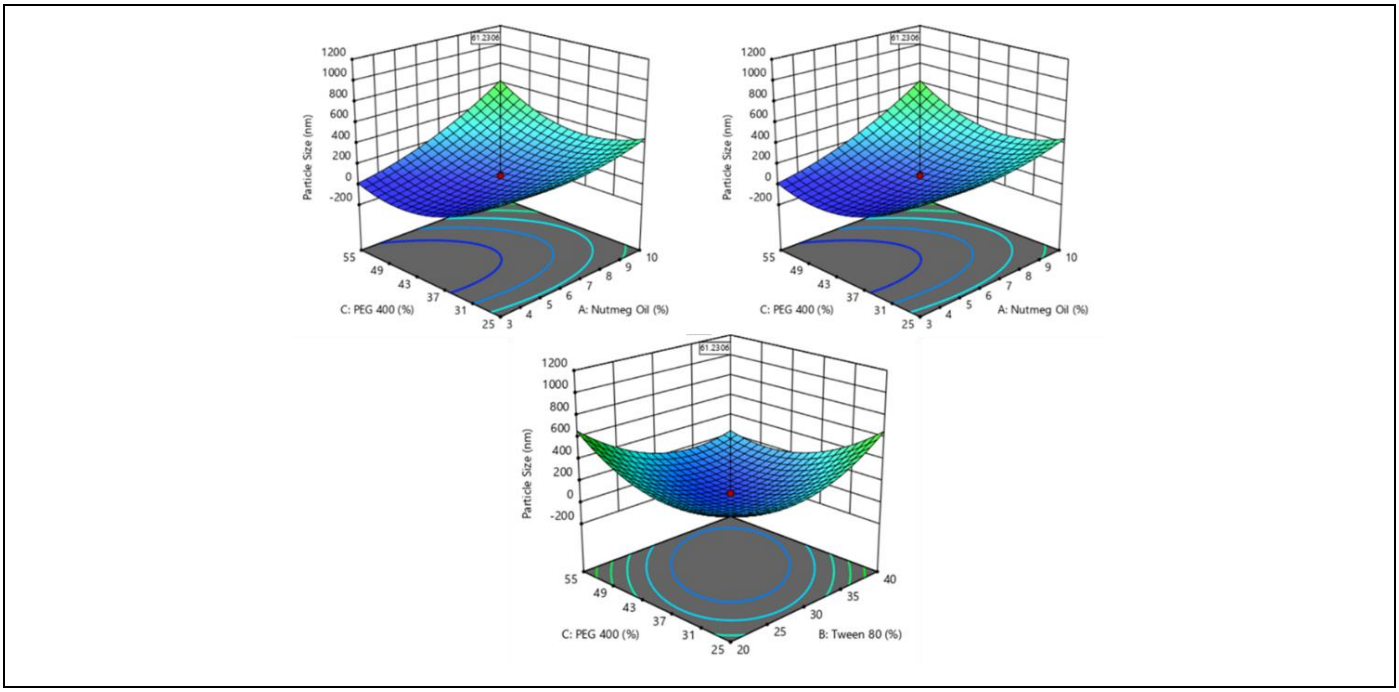

Figure 1. The 3D model interaction of independent variables with droplet size factor

3. RESULT AND DISCUSSION

The experimental data were utilized to compute the coefficients of the polynomial equation, which was subsequently employed to forecast the response values. The experimental values corresponded closely with the projected values derived from the RSM design. Table 3 displays the statistical model that characterizes the response surface for all responses. The models were considered appropriate if they were significant and yielded p-values below 0.05. Consequently, the results of this experiment indicated that the lack-of-fit test was not significant, with p values exceeding 0.05 and R2 and adjusted R2 values equal to or greater than 0.75. This model can be used to navigate the design space. This result is in line with the previous nanoemulsion optimization using CCD[31]. Based on the analysis of fit statistics, it can be concluded that the response of droplet size has a good modelling. The result of the observed and predicted value of particle size (Y1), zeta potential (Y2), polydispersity index (Y3) and transmittance percentage (Y4) can be seen in Table 4.

The model equation of droplet size response can be seen below:

$$Y1 = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B_2 + \beta_{33} C^2$$

$$Y1 = 61.23 + 180.14\,(A) - 44.80\,(B) - 37.65\,(C) - 103.82\,(AB) + 132.92\,(AC) - 172.75\,(BC) + 103.35\,(A^2) + 218.54\,(B^2) + 212.88\,(C^2) \quad (1)$$

Factor A (NMO) has a positive coefficient (+180.14), indicating that increasing the concentration of nutmeg oil leads to an increase in droplet size. This is because more oil raises the dispersed phase volume, which can resist droplet disruption during homogenization. This outcome aligns with other research indicating that an increase in oil may enhance droplet size[32]. The presented model demonstrated a linear escalation in droplet size with the enhancement of oil quantity. Research indicates that an increase in the dispersed phase (oil phase) can enhance flow resistance, potentially resulting in complications during the droplet disruption process. Factor B (Tween 80) has a negative coefficient (-44.8), meaning higher concentrations of Tween 80 reduce droplet size. Tween 80 stabilizes smaller droplets due to its strong interfacial activity. Factor C (PEG 400) also has a negative coefficient (-37.6), indicating that PEG 400 helps reduce droplet size by lowering interfacial tension. The interaction between Tween 80 and PEG 400 (BC) shows a strong negative effect (-172.75), suggesting that their combination synergistically reduces droplet size more effectively. The higher the Tween 80 and PEG 400 combination in the formula, the lower the droplet size. This result supports the conclusions of Yetukuri et al., suggesting that the model was statistically significant and adequate in demonstrating the genuine relationship between the response (droplet size) and the significant factors[33]. According to Miksusanti et al. the concentrations of Tween 80 and PEG 400 can affect droplet size[34].

Meanwhile, the AB interaction (NMO + Tween 80) has a negative coefficient (-103.8), also contributing to smaller droplets when both are present in higher amounts. The droplet size can decrease due to the

presence of the Tween 80 as a surfactant that may reduce the droplet size of the oil[35]. Conversely, the interaction between NMO and PEG 400 (AC) is positive (+132.92). It indicates that increasing of both components in nanoemulsion may lead to a larger droplet size. The mixture of PEG 400 and oil can enhance both the droplet size and the viscosity of the nanoemulsion[36].

The model highlighted the impact of Tween 80 concentration on droplet size. A reduction in Tween 80 corresponded with an increase in droplet size values. This may arise from the limited amount of surfactant leading to insufficient coverage of emulsifier molecules on the newly formed droplets[37]. PEG 400, as a cosurfactant, can significantly decrease interfacial tension, thereby reducing droplet size and enhancing stability[38]. Combining Tween 80 and PEG 400 can prevent product sedimentation. The use of Tween 80 in this study was because this surfactant has a longer fatty acid chain compared to Tween 60 and Tween 20[39]. The extended hydrophobic tail of Tween 80 enables it to solubilize greater quantities of poorly soluble compounds. The extended chain length enhances interactions at the oil-water interface, resulting in more stable emulsions that resist separation. This is particularly advantageous in compositions where long-term stability is essential[40]. The choice of PEG 400 was due to its compatibility with certain essential oils. The -OH groups present in PEG 400 facilitate the binding of NMO. The ability of glycol groups to bind essential oils can be attributed to the presence of intramolecular hydrogen bonds[41]. Thus, higher surfactant and cosurfactant levels shrink droplets, while higher oil content enlarges them.

The 3D model interaction of independent variables with droplet size factor can be seen in Figure 1. The model equation of zeta potential response (Y2) can be seen below:

$$Y2 = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B_2 + \beta_{33} C^2$$

$$\begin{aligned} Y2 = {} & -27.47 - 11.55\,(A) - 0.71\,(B) + 5.04\,C \\ & + 3.24\,(AB) - 7.41\,(AC) + 3.06\,(BC) \\ & - 6.03\,(A^2) - 12.98\,(B^2) - 13.10\,(C^2) \end{aligned} \quad (2)$$

Factor A (NMO) possesses a negative coefficient (-11.55), indicating that increased concentrations reduce the zeta potential value (which made it more negative). This could result in less stability as droplet aggregation may occur more easily. Adding oil components can increase the zeta potential in line with the droplet size increase so that the nanoemulsion's stability can decrease[23]. Factor B (Tween 80) in the zeta potential parameter only affects the response by (-0.71) yet factor C (PEG 400) affects the response by (+5.04), meaning that the higher the addition of PEG 400, the higher the zeta potential.

Previous studies' results stated that the addition of both Tween 80 and PEG 400 did not significantly impact the zeta potential value[42]. This event occurred due to Tween 80 and PEG-400, utilized in the formulation, being non-ionic surfactants devoid of charge on their hydrophobic groups[43]. A negative zeta potential in a

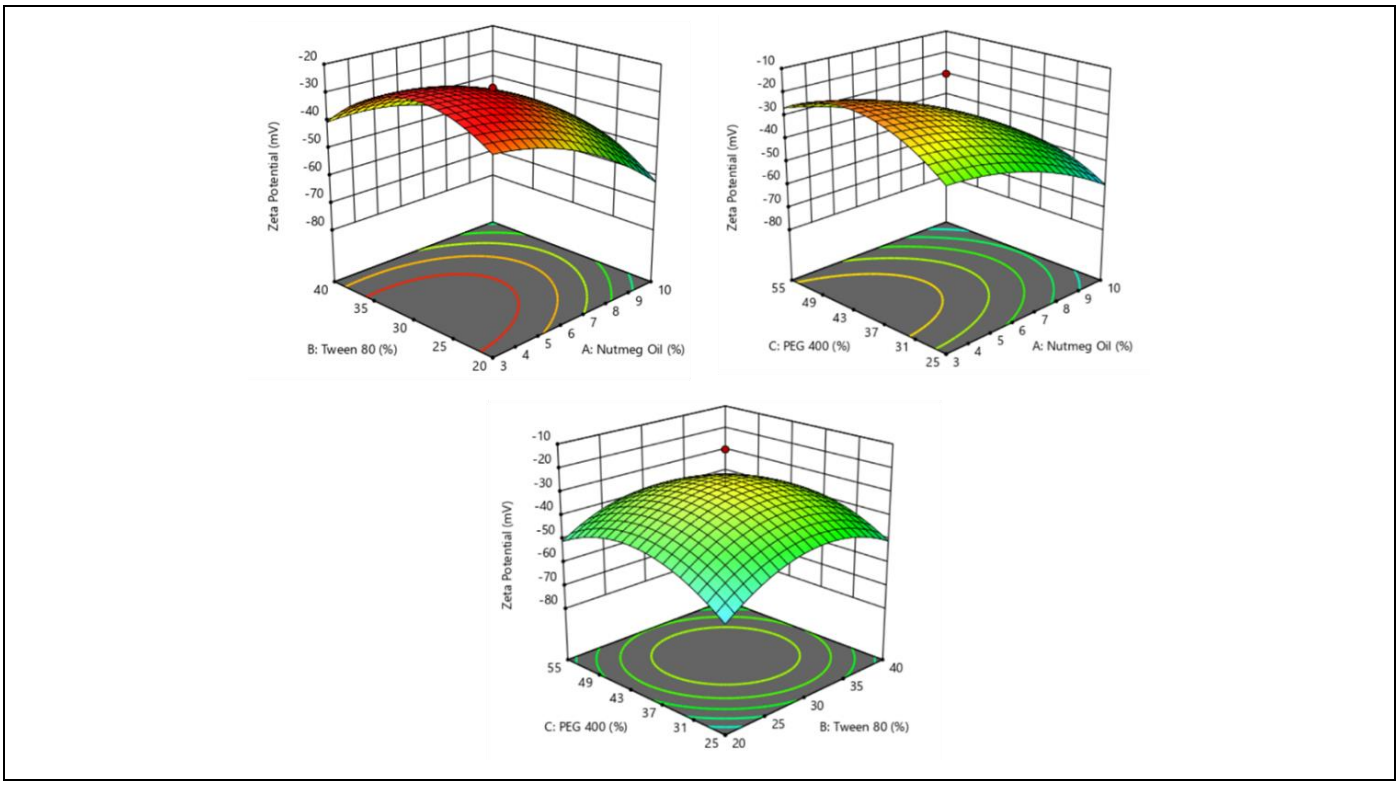

Figure 2. The 3D model interaction of independent variables with zeta potential

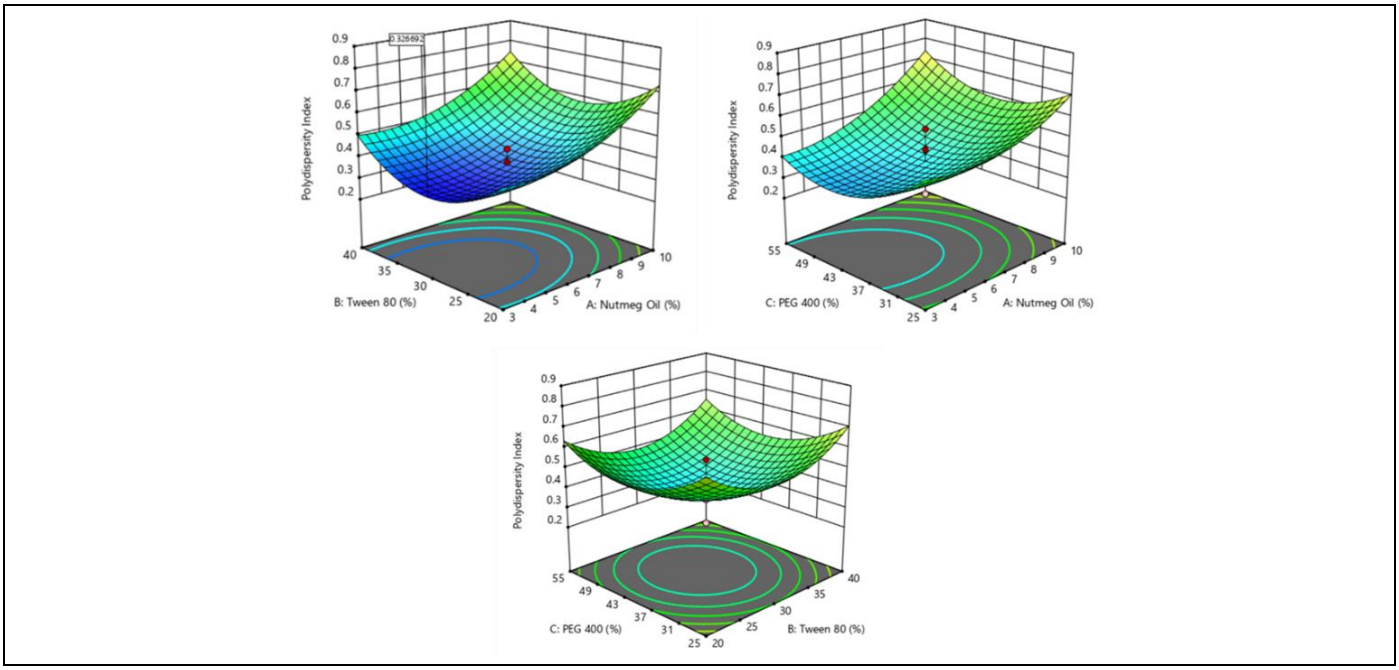

Figure 3. The 3D model interaction of independent variables with polydispersity index factor

nanoemulsion stabilized by a non-ionic surfactant signifies the existence of surface charge on the droplets, despite the surfactant being uncharged. The negative charge may result from the adsorption of ions from thesurrounding medium, including hydroxyl ions (OH-)[44]. Although non-ionic surfactants do not directly change the surface charge, their presence can modify the adsorption of ions, hence influencing the overall zeta potential[45]. The surfaces of the droplets coated with Tween-80 and PEG-400 are uncharged, as indicated by their low zeta potential. Reduced concentrations of Tween 80 in the formulation yield an elevated zeta potential. The zeta potential results approached 0 due to the use of non-ionic surfactants[46]. Overall, higher PEG 400 improves stability, while excess oil reduces it. The 3D model interaction of independent variables with zeta potential can be seen in Figure 2.

The zeta potential changes when NMO and Tween 80 are mixed together, increasing by +3.24, which means that using more of this combination in the formula can raise the zeta potential. The mix of NMO and PEG 400 (AC) affects the response by (-7.41), meaning that using more of both in the formula can lower the zeta potential. The combination of Tween 80 and PEG 400 (BC) results in an increase of +3.06; thus, incorporating greater quantitiesof both in the formulation will yield a more substantial enhancement in the zeta potential value. The interaction between oil composition and surfactant-cosurfactant significantly influenced the zeta potential compared to other interactions[47]. Nutmeg oil nano-emulsions and encapsulated forms often exhibit negative zeta potential values. An elevated zeta potential enhances repulsion among the droplets in the formulation, hence decreasing the likelihood of droplet aggregation.

The model equation of PI response can be seen below:

$$Y3 = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B_2 + \beta_{33} C^2$$

$$\mathrm{Y3} = 0.3839 + 0.125\,(\mathrm{A}) + 0.009\,(\mathrm{B}) - 0.028(\mathrm{C}) - 0.0021\,(\mathrm{AB}) + 0.043\,(\mathrm{AC}) + 0.006\,(\mathrm{BC}) + 0.084\,(A^2) + 0.16\,(B^2) + 0.14\,(C^2) \quad (3)$$

Factor A (NMO) influences the polydispersity index (PI) by +0.125, signifying that heightened NMO results in increased variability of droplet size. Factor B (Tween 80) in the PI response is influenced by +0.009, indicating that increased amounts of Tween 80 exert a negligible effect. Factor C (PEG 400) influences the response by -0.0281, indicating its role in lowering polydispersity. The combination of NMO and Tween 80 (AB) influences the response by (-0.0021), indicating that an increased concentration of NMO and Tween 80 in the formulation can diminish the PI value. The interaction of NMO and PEG 400 (AC) influences the response by (+0.0434), indicating that elevated concentrations of oil and PEG 400 enhance heterogeneity. The mixture of Tween 80 and PEG 400 (BC) influences the response by (+0.0006), indicating that this combination exerts a minimal effect on increasing the polydispersity index.

This result aligns with the findings of Gaber et al., who suggested that the addition of oil could potentially increase the polydispersity index[23]. The observed differences may arise from various factors, including the speed at which the emulsifier attaches to oil droplet surfaces, its ability to reduce interfacial

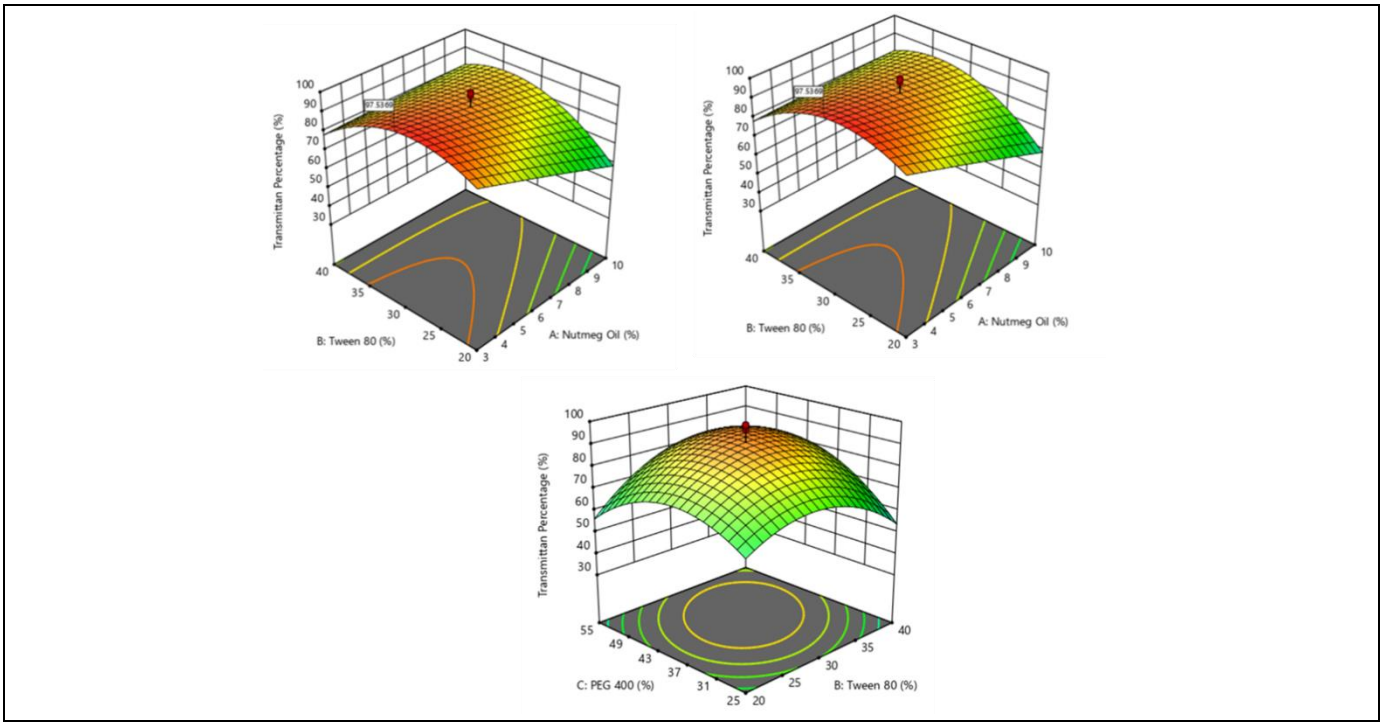

Figure 4. The 3D model interaction of independent variables with transmittance percentage factor

tension, and its effect on interfacial rheology[38]. The 3D model interaction of independent variables with polydispersity index can be seen in Figure 3.

The transmittance's percentage model equation can be seen below:

$$Y4 = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC + \beta_{11} A^2 + \beta_{22} B_2 + \beta_{33} C^2$$

$$Y4 = -44.24 - 6.92\,(A) + 2.73\,(B) + 3.86\,(C) + 8.20(AB) - 1.90(AC) + 7.65(BC) - 0.39\,(A^2) - 13.79\,(B^2) - 14.44\,(C^2) \quad (4)$$

Factor A (NMO) possesses a negative coefficient (-6.92), indicating that an increase in oil leads to lower transmittance, possibly due to larger droplet size and more light scattering. Factor B (Tween 80) has a positive coefficient (+2.73), suggesting that increased surfactant concentrations enhance clarity by producing smaller droplets. Factor C (PEG 400) positively influences transmittance (+3.86), suggesting that an increase in PEG 400 will enhance the transmittance percentage value. The combination of NMO and Tween 80 (AB) results in an increase of (+8.20) in responsiveness, signifying enhanced transparency. The response changes by -1.90 when NMO is combined with PEG 400 (AC). This signifies that the transmittance percentage falls when NMO is combined with PEG 400. Upon establishing the transmittance percentage in the upper range, the formula indicates that the combination of Tween 80 and PEG 400 (BC) may improve the clarity of the nanoemulsion. The transmittance percentage test was conducted to evaluate the droplet size and stability of the formulation, as any variation in transmittance percentage results in alterations to both droplet size and size distribution of the formulation[48]. This result was in line with Kumar et al. that TP is affected by the ratio of oil-surfactant-cosurfactant. Figure 4 showed the 3D model interaction of independent variables with transmittance percentage (TP).

The result of optimum formula (NECLX13) showed the desirability value of 0.874. Based on CCD, the desirability value close to 1 is chosen as the optimum formula. NECLX13 was selected as the optimum formula that consisted of consisted of 6.4% of NMO, 33.7% of Tween 80 and 43% of PEG 400. The result of predicted value and observed result of optimum formula are shown in table 4. The actual result of the optimum formula showed similarity to the predicted result. NECLX13 demonstrated significant correlation between observed and projected values for all critical response parameters, including droplet size, zeta potential, polydispersity index, and transmittance. The

Table 5. The organoleptic, pH, viscosity and phase separation test result of the optimum formula (NECLX13)

Organoleptic Parameters	NECLX13
Visual inspection	Transparrent and yellowish
pH	6.77 ± 0.12
Viscosity	122.5 ± 0.15
Phase separation	Not detected

Figure 5. The organoleptic result of NECLX13

slight differences validate the model's precision and resilience. The results, along with the enhanced penetration and anti-inflammatory efficacy of NECLX13, confirm its designation as the optimal formulation. The organoleptic result of NECLX13 can be seen in table 5. The result showed that the NECLX13 had a clear-yellowish color. The viscosity and pH was in line with the previous study and it did not show any separation[24]. The organoleptic result of NECLX13 can be seen in Figure 5.

The microscopy image of the optimum formula of CLX nanoemulsion can be seen in Figure 6. The microscopy image showed the droplet size was 50 nm. This result is in line with Mardiyanto et al. that nanoemulsion had the droplet size below 200 nm[10]. Tween 80 combined with PEG 400 can reduce the globule size and increase the stability of nanoemulsion.

The *in vitro* permeation result of CLX-NE optimum formula can be seen in Table 6. The optimized CLX-NE formula (NECLX13) with the highest flux was the most effective. This is due to the nanoemulsion droplets' small size, which facilitates the preparation's passage through the epidermis membrane. PEG 400 functions as an enhancer and can increase the solubility of substances that are difficult to dissolve in water. PEG 400 is recognized for its capacity to combine with NMO and generate a transparent solution[49] The results on the optimum formula showed a higher flux compared to CLX + PEG 400 and CLX + Tween 80. Surfactants reduce interfacial tension and cosurfactants hold excess water phase through hydrogen bonds[50]. The reduction of the droplet size enhanced the likelihood of obtaining an optically clear nanoemulsion with increasing surfactant content. Tween 80 may enhance the fluidity of intercellular lipids and facilitate the permeation of the active ingredient into the dermis. The substantial droplet size arising from phase behavior may contribute to an optically opaque nanoemulsion. Multiple formulations in the investigation, including NECLX4, NECLX7, and NECLX8, displayed an opaque appearance, attributable to their considerably larger droplet sizes (about 700 to over 1100 nm) and elevated polydispersity indices. These characteristics lead to increased light scattering, which lowers transmittance values and suggests a coarser, less stable emulsion structure. Conversely, NECLX13 exhibited a very clear look with a transmittance of 98.9%, attributable to its small droplet size (45.7 nm) and low polydispersity index, consequently affirming its designation as a genuine nanoemulsion. This highlights the significance of optimized surfactant-co-surfactant-oil ratios in attaining nanoscale droplet production and improved formulation stability.

NMO is significant in the permeation investigation because of its composition. It contains of oleic acid and palmitic acid. Quinones et al. report that CLX combined with oleic acid and glycerol mono-oleate can reduce ear edema in rabbits by 53.5% more effectively than CLX itself[50].

According to the findings of the anti-inflammatory test based on inhibition percentage of paw edema data in table 7, the optimal formulation can efficiently minimize paw edema. The *in vivo* anti-inflammation result of CLX in various formulations can be seen in Figure 7. The *in vivo* anti-inflammation test of CLX-NE with paw-edema method before induction (a) and after induction (b) with 1% carrageenan solution can be seen in Figure 8. The optimal formulation is capable of preserving the normal paw of the rats for six hours. When compared to the results of other groups, the NECLX13 was significantly different ($p = 0.012$). The percentage of inhibition of CLX in PEG 400 and

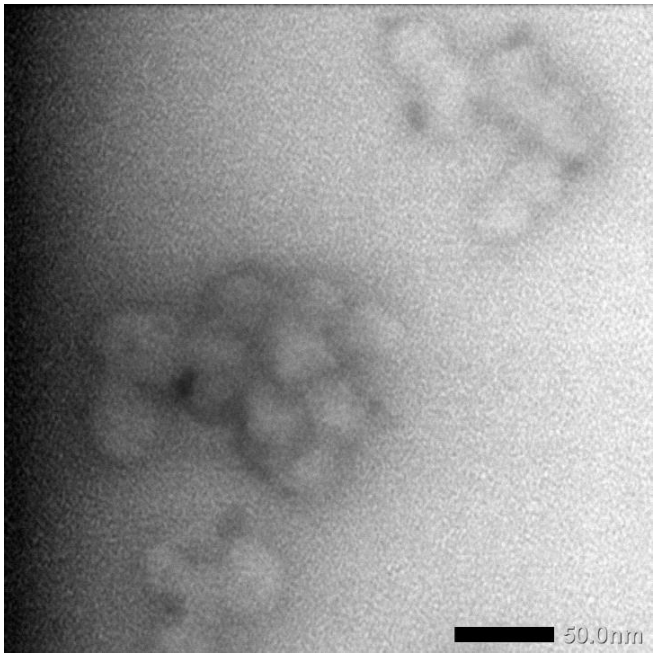

Figure 6. The microscopy image of the optimum formula of CLX nanoemulsion

Table 6. The result of in vitro permeation study of optimum formula of CLX nanoemulsion

Group	Flux (mg.cm^2/h)
NECLX13	60.233 ± 2.146^{b,c,d}
CLX+NMO	41.237 ± 2.135^{a,c,d}
CLX+Tween 80	24.721 ± 1.251^{a,b}
CLX+PEG 400	25.341 ± 1.375^{a,b}

Data displayed: n=3 replication tests ± standard deviation
a= significantly different to NECLX13 c= significantly different to CLX+Tween 80
b= significantly different to CLX+NMO d= significantly different to CLX+PEG 400

CLX in Tween 80 was identical (p = 0.088), which indicates that both products have the same ability to alleviate paw edema. Untreated group showed the least of inhibition percentage followed by NE base. This result showed that the NE base did not give any effect on the paw edema.

This in vivo test result also showed that globule size is related to skin permeation as the smaller globule size, the more effective topical delivery[51]. Voltaren Emulgel® was used as the positive control that contains 1% diclofenac diethylamine. The use of this marketed product is due to its effect as an anti-inflammation that also inhibits COX and it is used as one of topical rheumatoid arthritis treatment in Indonesia[52]. NECLX13 showed the same inhibition percentage as the Voltaren Emulgel®. In this case, NMO as a carrier oil also helps in reducing paw edema. It has the ability to inhibit COX-2, which enables it to perform the functions of both an anti-inflammatory and a pain reliever[53,54]. This occurs due to the fact that NMO contains oleic acid and myristicin. It was found that myristicin had a substantial impact on the production of calcium, nitric oxide (NO), interleukin (IL)-6, IL-10, interferon inducible protein-10, monocyte chemotactic protein (MCP)-1, MCP-3, granulocyte-macrophage colony-stimulating factor, and macrophage inflammatory protein[54,55].

This result suggested that nanoemulsion could be a promising method for the topical delivery of CLX. The *in vitro* and *in vivo* tests NECLX13 were incomparable due to the absence of the marketed CLX topical product in Indonesia. The stability assessment of CLX-NE may be conducted based on the results of this investigation.

4. CONCLUSIONS

A central composite design was effectively used to generate and optimize an CLX nanoemulsion with surfactant, cosurfactant and NMO as independent variables, which enhanced the drug's skin penetration. The optimized CLX-NE (NECLX13) formula demonstrated a droplet size of 48.30 ± 1.30 nm, with % transmittance at 95.22 ± 1.15, zeta potential at -29.30 ± 2.41 mV, and polydispersity index at 0.488 ± 0.015, respectively. The optimized CLX-NE formulation

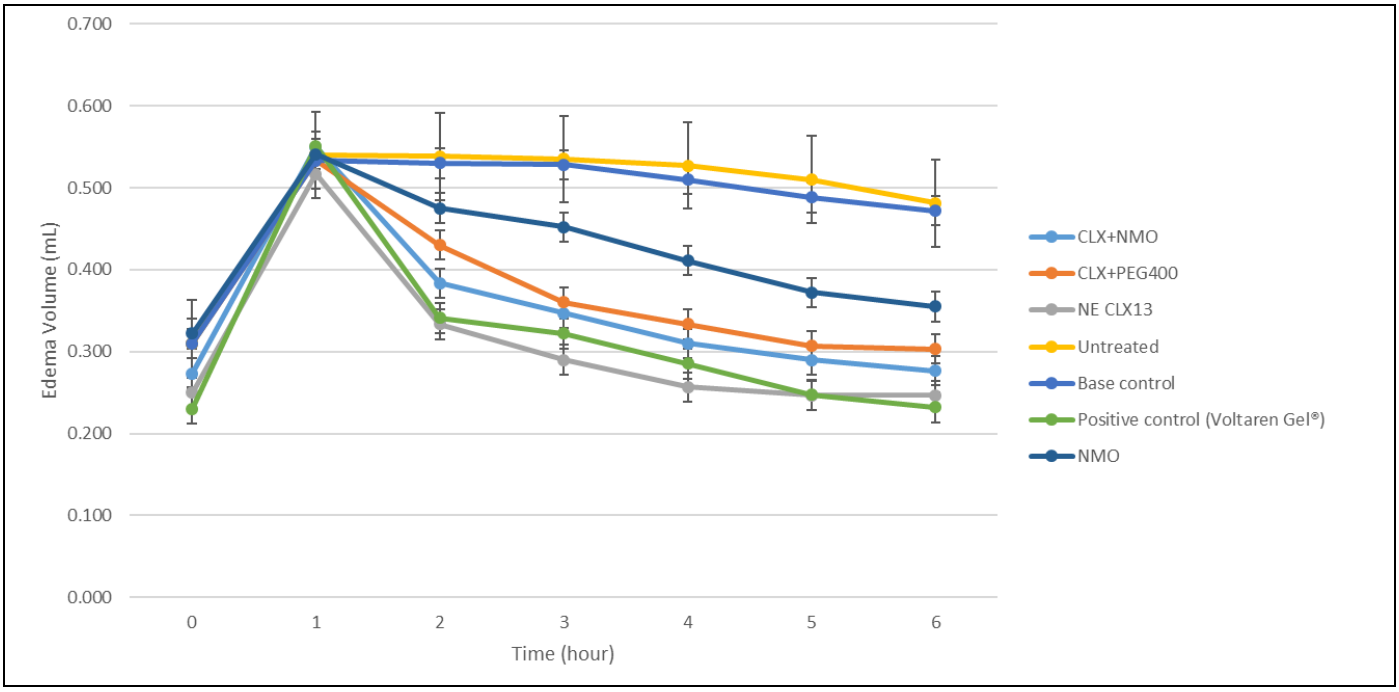

Figure 7. The *in vivo* anti-inflammation result of CLX in various formulations

Table 7. The result of inhibition percentage of in vivo anti-inflammation result of CLX in various formulations

Formula	Percent of Inhibition ± Standard Deviation
NECLX13	56.87±2.31[a,b,c,e,f]
CLX+NMO	49.79±2.35[b,c,d,e,f,g]
CLX+Tween 80	33.82±3.12 [a,d,e,f,g]
CLX+PEG 400	39.65±2.85[a,d,e,f,g]
Convensional Emulsion	25.40±2.74 [a,b,c,d,f,g]
NE base (Tween 80 + PEG 400)	11.55 ± 1.44 [a,b,c,d,e,g]
Voltaren Emulgel®	57.89±2.81 [a,b,c,e,f,g]

Data displayed (n) = 6; a= significantly different to CLX+NMO; b= significantly different to CLX+PEG 400; c= significantly different to CLX+Tween 80; d= significantly different to NECLX13; e= significantly different to convensional emulsion; f = significantly different to NE base; g = significantly different to Voltaren Emulgel®

demonstrated a drug release that was greater than that of the drug itself in surfactant or co-surfactant and carrier oil. The NECLX13 can reduce the rats' paw edema within 30 minutes and maintain the edema until 6 hours. The observed results have paved the way for further research, during which pharmacokinetics and pharmaco-dynamics studies will be undertaken to validate the *in vivo* potential of the drug. In conclusion, this study successfully optimized CLX-NE through a central composite design, highlighting its potential to enhance the flux and the skin permeation of CLX for topical preparation.

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Author contribution

AS: Concepts or ideas; design; literature search; experimental studies; data analysis; manuscript preparation
MFR: Experimental studies; software operating; literature search; data analysis
DNW: Experimental studies; literature search; data analysis
JH: visualization, literature search
AR: Experimental studies; data curation
NSR: Experimental studies; data curation
YNW: Manuscript editing; manuscript review
MZS: Manuscript editing; manuscript review
SM: Manuscript editing; manuscript review.
All authors have read and agreed to the published version of the manuscript.

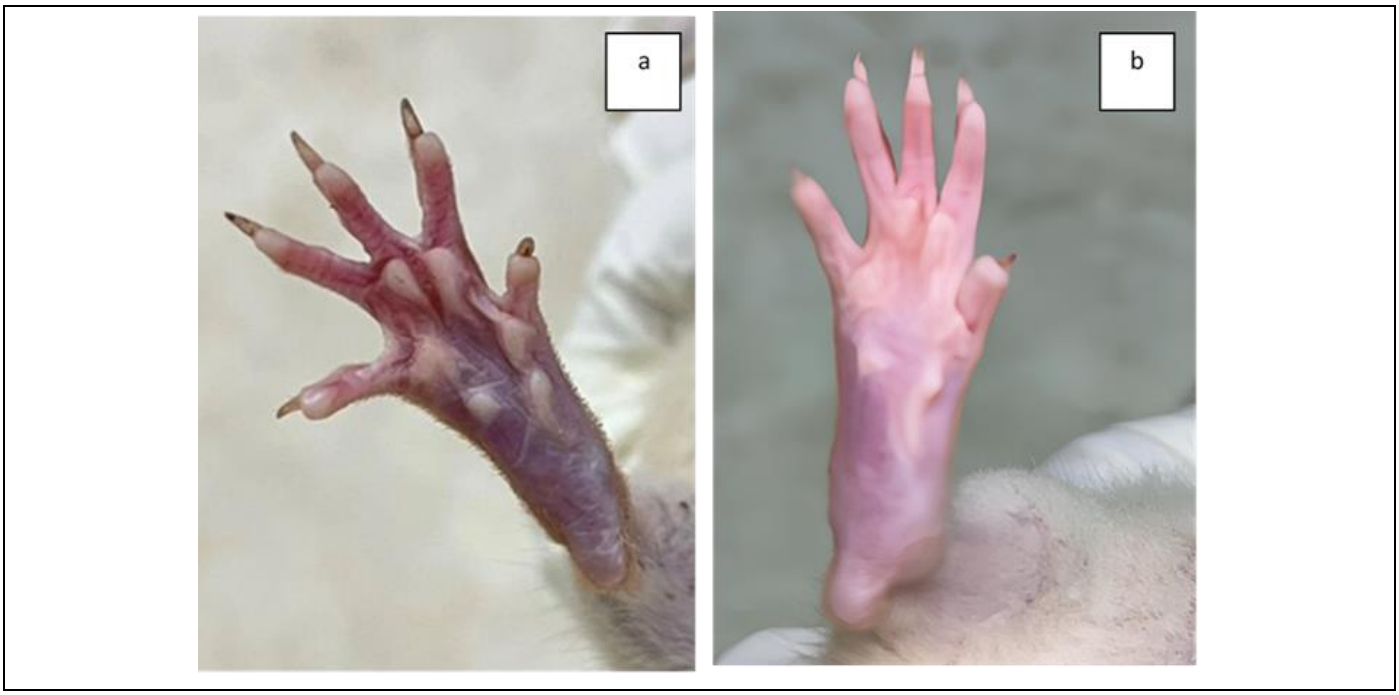

Figure 8. The *in vivo* anti-inflammation test of CLX-NE with paw-edema method before induction (a) and after induction (b) with 1% carrageenan

Conflict of interest (If any)
None to declare.

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Ethics approval
The *in vivo* test had been approved by the ethics commission of Health Research Ethics Committee of Ministry of Health of Health Polytechnic with number of 1368/EA/F.XXIII.38/2024.

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