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Formulation development and *in vivo* study of nanoemulgel of *Channa striata* and *Citrus limon* extract for caesarean wound treatment

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ABSTRACT

Empirically, snakehead fish with its high albumin content has been widely used in the healing of post-caesarean wounds. Lemon can also be used to prevent wound infections by inhibiting thegrowth of bacterial activities. This study was conducted to discover the best nanoemulgel formula from the combination of snakehead fish and lemon extract, then evaluate its healing activity for post-caesarean wounds. Nanoemulgel was characterized by pH, viscosity, spreadability, adhesion, particle size, and zeta potential to determine the best formula. Healing activity evaluation was performed on female Wistar rats which were divided into seven groups (n=3). Healing parameters evaluated were wound length closure, epidermal thickening, and tissue reconstruction. Wound length was measured every 5 days for 15 days. Subsequently, histopathological observations H&E staining were used to determine epidermal thickening and wound tissue reconstruction. The results showed that nanoemulgel I (NEG1) containing 7% snakehead fish extract, and 3% lemon extract had the best wound healing ability, with an average wound closure of 84±2.1% on day 10, epidermal thickening of 2.31 ± 0.06 µm on day 15, and better tissue structure reconstruction than other groups. Thus, the nanoemulgel design can optimize

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the snakehead fish and lemon extract in accelerating the healing process of post- caesarean wounds.

Keywords: *Channa striata*, *Citrus limon*, nanoemulgel, reepithelialization, wound healing

INTRODUCTION

According to the World Health Organization (WHO), maternal perinatal mortality and morbidity can be prevented through caesarean section, with a percentage ranging from 10% to 15%[1,2]. The Cesarean Section Rate (CSR) initially was 5%, then increased to 12% in 2012, and further rose to 17% in 2017 in Indonesia. Ayuningtyas et al. (2018) reported that caesarean sectionhas become a trend, constituting up to 70% of deliveries in private hospitals[3]. The prevalence of pain and morbidity in mothers after caesarean section is higher compared to vaginal deliveries because it can lead to infections, the most common complication[4–6]. Although antibiotics are widely available to prevent infections, the risk of infection continues to rise[7,8]. Infections in post-caesarean operation wounds can be prevented by accelerating the reepithelization process[9].

The speed of the reepithelization process can be increased by elevating the albumin levels inthe body[10-12]. Therefore, the majority of mothers after caesarean section are recommended to consume snakehead fish due to its high albumin content[13]. This is considered less effective because it does not directly reach the target action, resulting in suboptimal efficacy. Currently, snakehead fish albumin is widely developed in capsule form, but oral administration has low bioavailability, need to undergo first-pass metabolism, chemical degradation, and enzymatic reactions, therefore topical administration is more recommended[14]. Therefore, Andrie and Sihombing used 5-10% snakehead fish extract on ointment formulation for wound healing. However, the healing acitivity only reached the inflammation stage[15]. Besides using albumin, the acceleration of healing in post-caesarean operation wounds can be assisted by using citric acid because it inhibits the growth of bacterial activities[16,17]. Sim et al. have proven that 3% citric acid has wound healing potential[18]. An acidic environment significantly influences wound healing as it acts as a natural physiological response to mediate various cellular processes internally to restore barriers which facilitate higher oxygenation levels in the wound, affect macrophage and fibroblast activities, as well as enzymatic activities which participate in wound healing[18].

The advancement in drug synthesis has led to the development of nanocarriers as a strategyto achieve efficient drug penetration, making them suitable for a topical drug delivery system. Some researchers have claimed that nanoemulsions are a potential drug delivery system due to their high drug loading, solubility capacity, ease of manufacturing, stability, and controlled releasepatterns[19,20]. However, nanoemulsions have low viscosity, resulting in brief contact with the skinand suboptimal penetration of active substances. Therefore, to obtain an effective topical formulation, a nanoemulgel formulation was created to extend the contact time of the formulationwith the skin, providing high drug loading, penetration, and better diffusion compared to other formulations[21,22].

METHODOLOGY

Materials

Snakehead fish (*Channa striata*) and lemon (*Citrus limon*) were purchased from the local market in Solo, Central Java. Materials and solvents used for extraction included distilled water obtained from PT. Agung Jaya (Solo, Central Java), ether, sodium sulfite, sodium acetate, buffer solution, and $CaCl_2$ obtained from Merck (Darmstadt, Germany), then HCl 37%, H_2SO_4, NaOH 6M purchased from CV. Anugrah Jaya Kimia (Surabaya, East Java). Determination of albumin and citric acid content in the extract used standard bovine serum albumin (BSA) from Sigma Aldrich, biuret reagent (ROFA Lab), methylene blue, Sudan III, and citric acid anhydrous 99% from Merck (Darmstadt, Germany), deionized water (OneMed, Indonesia), and technical grade ethanol from PT. Agung Jaya (Solo, Central Java). The formulation of nanoemulsion and nanoemulgel used Tween 80, Propylene glycol, and olive oil from Merck (Darmstadt, Germany), carbopol 940 and triethanolamine (TEA) purchased from Petronas Chemical, Malaysia, and Dimethyloldimethyl (DMDM) hydantoin purchased from CV. Cipta Kimia (Sukoharjo, Central Java).

Methods

Snakehead fish extraction and albumin content determination

A total of 50 grams of finely minced snakehead fish meat was mixed with 125 ml of acetatebuffer solution, then centrifuged at a speed of 2500 rpm for one hour. The snakehead fish solutionwas separated from the meat sediment, and then 150 ml of ether and 30 ml of 25% sodium sulfitewere added. It was centrifuged again at 2500 rpm for two hours. The sediment (the bottom layercontaining albumin) was separated from the supernatant (the upper layer) for analysis of the albumin content contained within it. The analysis of albumin levels in snakehead fish extract wasusing spectrophotometry with a wavelength range of 450-700 nm. The materials required for analysis include a standard albumin, BSA (bovine serum albumin), and biuret reagent[23].

Lemon extraction and citric acid content determination

A total of 75 ml of lemon juice was adjusted to a pH of 7.5-8 by adding 2M NaOH. Then, 37.5 ml of 10% $CaCl_2$ was added to the lemon juice, and it was heated on a hotplate until it reached a constant boiling temperature of 165°C. Subsequently, the solution was decanted, and the formedprecipitate was treated with 8.25 ml of 2M H_2SO_4. This mixture was heated and decanted again, similar to the previous process. The obtained precipitate was left to dry[24]. The analysis of citric acid levels in lemon extract was using spectrophotometry with a wavelength range of 190-280 nm. The materials required for analysis include citric acid standard, deionized water, and 0.25 Mof HCl[24,25].

Preparation of snakehead fish and lemon extract nanoemulsion

Two formulations of nanoemulsion (NE) with snakehead fish and lemon extracts were developed. NE1 consists of 30% propylene glycol, 25% tween 80%, 7% snakehead fish extract, 3% lemon extract, 5% olive oil, and distilled water. NE2 differs in the percentage of the two extracts, with 3% snakehead fish extract and 7% lemon extract. The homogenization anddispersion of nanoemulsion particles were carried out using a magnetic stirrer and ultra turrax at a speed of 12,000 rpm for 5 minutes[26].

Characterization of snakehead fish and lemon extract nanoemulsion

Nanoemulsion (NE) characterization was tested based on emulsion type criteria, kinetic andthermodynamic stability, pH, and particle size[27,28].

Preparation of gel base and incorporation of nanoemulsion in the gel base

The gel base is prepared by dispersing 2% carbopol in warm water, followed by adding 1.15% triethanolamine (TEA), 0.38% dimethyloldimethyl (DMDM) hydantoin, and aquadest to make a total of 100 grams of gel base. The incorporation of 25 grams of NE into 75 grams of thegel base is achieved through a combination of an ultra turrax at 10,000 rpm for 5 minutes and anultrasonicator probe with a 70% amplitude for 45 minutes[29].

Characterization of snakehead fish and lemon extract nanoemulgel

Nanoemulgel (NEG) characterization was tested based on pH, viscosity, spreadability, adhesion, particle size, and zeta potential[27,28,30].

In vivo study for wound healing activity of snakehead fish and lemon extract nanoemulgel

A total of 21 female rats aged 2-3 months underwent acclimatization for two weeks in the Animal Laboratory of Faculty of Medicine UNS. The rats were then divided into seven treatmentgroups. Post-operative wounds were created through minor surgery on the abdominal skin of therats with an incision length of ±2 cm, stitched using plain catgut. After surgery, the rats were treated according to the assigned test group, with Group I as a placebo control, II with NEG1, III with NE2, IV as normal, V with commercial preparation, VI with NEG1 extract, and VI with NEG2 extract. The treatment was given for 15 days, and wound length examination was every 5 days[31].

Histopathology examination of wound tissue

Skin tissue from the abdomen, approximately 3x3 cm in size, was collected, washed with sodium chloride solution, and then immersed in 10% Neutral Buffered Formalin (NBF). The tissue was then stained with H&E (Hematoxylin and Eosin). Wound re-epithelialization was observed under a light microscope at 100× magnification[18].

Data analysis

The analysis methods involved the use of software such as MS Excel, SPSS, GraphPad Prism,and Image-J. The wound tissue re-epithelialization was analyzed with Image-J and the effectivityof the gel in wound healing was concluded through One-Way ANOVA analysis with a confidencelevel of 95%.

RESULTS and DISCUSSION

Determination of albumin content in snakehead fish extract

The extraction of snakehead fish (SF) was performed at the isoelectric point (pH 4,6) which is the pH range when protein solubility is lowest, making it easier to form precipitates[32]. Therefore, a pH 4.6 acetate buffer was used as the solvent. Separation of the extract from the SFmeat residue was carried out by centrifugation for one hour at 2500 rpm. Purification of the SF extract from non-protein components was done using ether with the "like dissolve like" principle.The precipitation of the SF extract containing albumin (AL) was achieved through the salting-out mechanism by adding sodium sulfite[33]. The yield of the extraction process shown in Table 1. Thepresence of AL content in SF extract was confirmed through the similarity of wavelengths and visible spectrum between the SF extract and BSA[34]. The maximum wavelength for BSA is 544 nm and the maximum wavelength for the extract sample is 543 nm, with the similiar spectrum asshown in Figure 1(a). Quantification showed that there was 77% AL content in the yield of the SFextract.

Table 1. Yield of the snakehead fish extraction

Sample	Weight (g)	Yield (%)
Snakehead fish meat	50	-
Snakehead fish extract	0.4	0.8
Albumin content	0.3	77

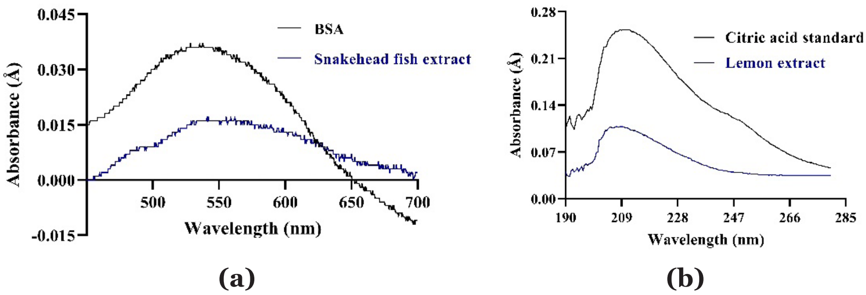


Figure 1. Visible spectrum of Bovine Serum Albumin (BSA) and snakehead fish extract (a); UV spectrum of citric acid standard and lemon extract (b)

Determination of citric acid content in lemon extract

The lemon extraction process begins by creating lemon juice in an alkaline environment through the addition of sodium hydroxide to form clearer and amorphous crystal precipitates[35]. Citric acid (CA) in the lemon reacts with the added calcium chloride, resulting in the formation of a salt precipitate. At this stage, two salts are formed: calcium citrate, which precipitates, and sodium chloride, which remains dissolved. Therefore, decantation is performed to separate them.The calcium citrate precipitate is then converted back into CA by adding sulfuric acid[24]. The yieldof the extraction process shown in Table 2. The presence of CA content in lemon extract was confirmed through the similarity of wavelengths and UV spectrum between the lemon extract andCA standard[34]. The maximum wavelength for CA standard is 210 nm and the maximum wavelength for the extract sample is 209,5 nm, with the similar spectrum as shown in Figure 1b.Quantification showed that there was 64% CA content in the yield of the lemon extract.

Table 2. Yield of the lemon extract

Sample	Weight (g)	Yield (%)
Lemon juice	75	-
Lemon extract	3.75	5
Citric acid content	2.39	64

Characterization of snakehead fish and lemon extract nanoemulsion

Nanoparticles (140-400 nm) can optimize the delivery of active ingredients due to their large particle surface area, allowing for an increased number of particles that can be incorporated into the hydrogel matrix[19,36]. Based on Table 3, the particle size of both formulations fell within the nanoparticle range, with 294.13 ± 1.63 nm for NE1 and 181.5 ± 0.78 nm for NE2. The particle size distribution of both formulations can be seen in Figure 2 and is classified as a monodisperse andhomogeneous system (PDI < 1.0) with a PDI value of 0.39 ± 0.01 for NE1 and 0.24 ± 0.03 for NE2[37].

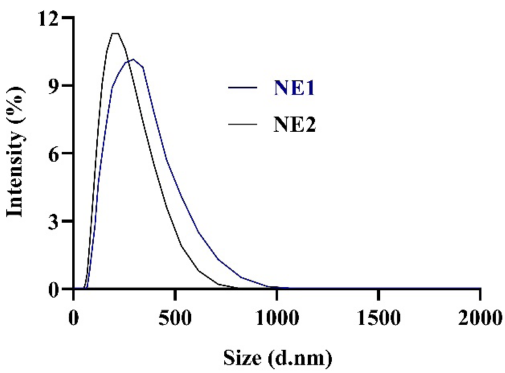


Figure 2. Particle size distribution of nanoemulsion formulations

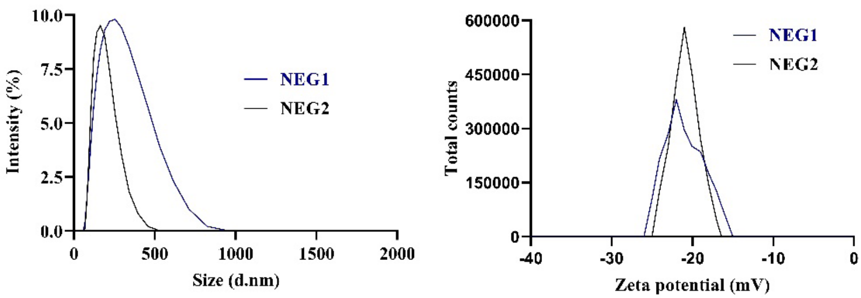
The higher content of citric acid in NE2 compared to NE1 makes the pH of NE2 more acidic than NE1, as presented in Table 3. Both formulations show that they are O/W emulsion type which are more stable for incorporation into water-based gel formulations. Both NE formulationsare stable kinetically because after the kinetic stability test with centrifugation at 3800 rpm for 30 minutes, no creaming or flocculation occurred[38]. However, both NE formulations exhibited thermodynamic instability, as indicated by the reversible separation of the aqueous and oil phasesafter storage at -4°C and 40°C for 24 hours[39]. This is consistent with the findings of Ullah et al. that NE tend to be thermodynamically unstable but stable kinetically[27].

Table 3. Characterization of the snakehead fish and lemon extract nanoemulsion formulations (Mean ± SD, n=3)

Formulation code	pH	Emulsion type	Mean particle size (nm)	Polydispersity index	Stability	
					Kinetic	Thermodynamic
NE1	3.36 ± 0,09	M/A	294.13 ± 1.63	0.39 ± 0.01	√	√
NE2	2.69 ± 0,1	M/A	181.5 ± 0.78	0.24 ± 0.03	√	√

Characterization of snakehead fish and lemon extract nanoemulgel

Both nanoemulgels (NEGs) were within the nanometer particle size range that can easily penetrate the skin, which is 140-400 nm[36]. The droplet size for NEG1 and NEG2 are 223,27 ± 8,02 nm and 231,80 ± 10,58 nm with monodisperse system (PDI < 1,0)[37], as presented in Figure 3a. Figure 3b shows the zeta potential values of NEG1 and NEG2 are -22.4 ± 0.4 and -21.2 ± 0.6, indicating good stability (-30 mV hingga -20 mV)[40,41].



(a) (b)

Figure 3. Particle size distribution of nanoemulgel formulations (a); zeta potential distribution of nanoemulgel formulations (b)

As presented in Table 4, the pH values of both formulations fall within the safe pH range forskin (5,0-8,0)[42]. NEG1 has higher viscosity than NEG2, indicating that NEG1 provides better adhesion, prolonging the contact time of the formulation with the skin[43]. On the other hand, NEG2, with lower viscosity, exhibits better spreadability compared to NEG1[44].

Table 4. Characterization of the snakehead fish and lemon extract nanoemulgel formulations (Mean ± SD, n=3)

Formulation code	Mean particle size (nm)	Polydispersity index	Zeta Potensial (mV)	pH	Viscosity (cPs)	Spreadability (cm)	Adhesiveness (s)
NEG1	223.27 ± 8.02	0.5 ± 0.04	-22.4 ± 0.4	6.83 ± 0.03	8038 ± 3	3.20 ± 1.42	17.41 ± 2.41
NEG2	231.80 ± 10.58	0.66 ± 0.01	-21.2 ± 0.6	6.77 ± 0.04	445 ± 3	4.30 ± 3.78	2.07 ± 0.78

In vivo wound healing activity

The wound healing pattern in post-operative rats in the in vivo study, as shown in Figure 4, illustrates the ability of the NEG formulation of SFL extract to aid in the healing of post-caesareansection wounds. Wound healing activity is assessed by the closure of the wound and the fading of scars.

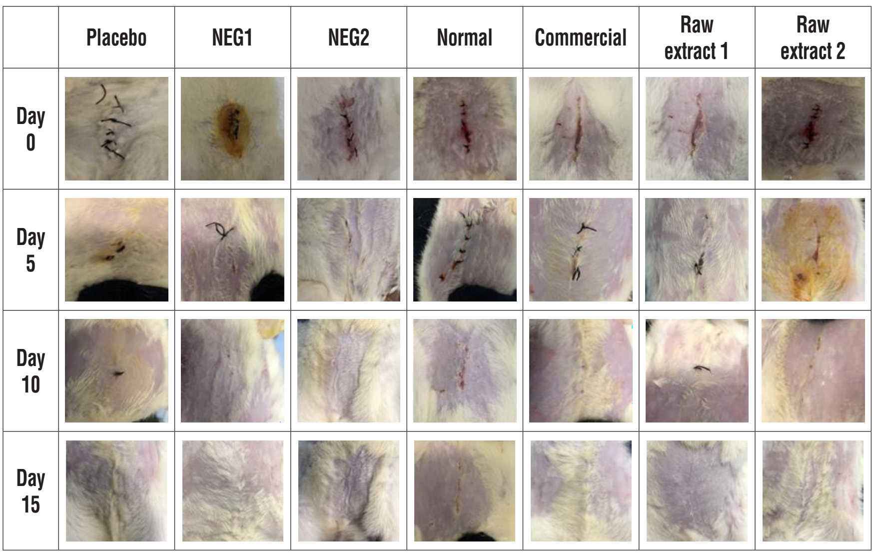


Figure 4. Representative image of wound healing progress in rat for 15 days

Furthermore, the length of wound closure was calculated every 5 days for all treatment groups, and the results are presented in Figure 4. Meanwhile, Figure 5(a) shows the percentage ofwound closure for all groups on the 10th day, which is significantly different (p-value < 0.05). Thegroups treated with NEG1 and NEG2 also exhibited significantly different percentages of woundclosure compared to the normal group, indicating that the NEG formulation of lemon and snakehead fish extracts has wound healing activity. NEG1 and NEG2 are also found to have betterwound healing activity than commercial albumin formulations because the percentage of woundclosure in both of these groups significantly differs from the commercial formulation group. The percentage of wound closure in the NEG1 and NEG2 groups also significantly differs from the NEG1 extract and NEG2 extract groups, indicating that the NEG carrier can optimize the effectiveness of SFL extracts in wound healing. The NEG1 group differs significantly from the NEG2 group, with an average percentage of wound closure (%) in the NEG1 group of 84 ± 2.1 andin the NEG2 group of 81 ± 1.5. It can be concluded that NEG1 has better wound healing activity than NEG2.

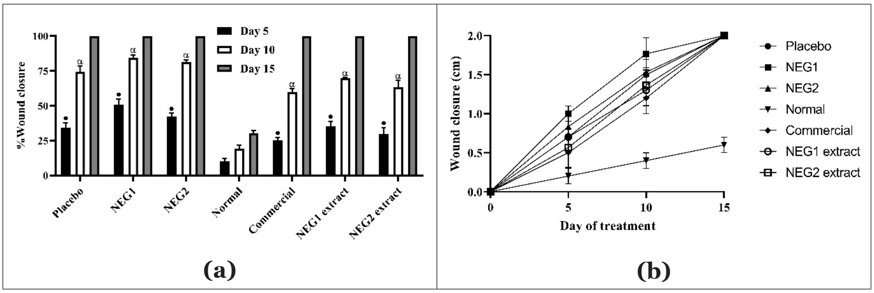


Figure 5. Wound closure percentage diagram (a); wound closure length from day 0 until day 15 (b). The significant difference (p<0,05) in comparison with normal group are expressed as • for day 5 and α for day 10.

Histopathology of wound tissue

Epidermal thickening accompanied by an abundance of sebaceous glands and hair follicles indicates better tissue regeneration of the skin[45]. Figure 6(c) shows that on the 15th day, the NEG1group has mature hair follicles, numerous sebaceous glands, and a more organized skin structure compared to the other groups. The placebo group, NEG2, commercial group, NEG1 extract, and NEG2 extract also displayed sebaceous glands and hair follicles, as seen in Figure 6. However, the skin structure and the maturation of the glands were not as advanced as in the NEG1 group. In the normal group, only undifferentiated glands were present.

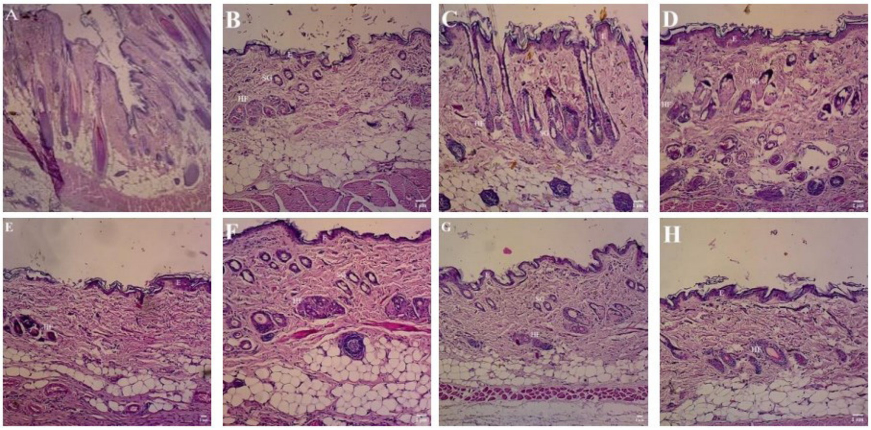


Figure 6. H&E staining photomicrographs of all groups: wound skin (A), placebo group (B), NEG1 group (C), NEG2 group (D), normal group (E), commercial product group (F), NEG1 extract group (G), and NEG2 extract group (H). Epidermis (E), sebaceous glands (SG), and hair follicles (HF) were marked.

Figure 7 illustrates the epidermal thickness from largest to smallest as NEG1 > NEG2 > Commercial > NEG1 extract > NEG2 extract > Placebo > Normal. The test results showed that only the epidermal thickness of the NEG1 and NEG2 groups differs significantly (p-value < 0.05)from the normal group. Therefore, it can be inferred that NEG1 and NEG2 are more effective in aiding wound healing compared to others. The epidermal thickness of NEG1 significantly differs from NEG2, with an average epidermal thickness of the NEG1 group at 2.31 ± 0.06 µm and the NEG2 group at 1.69 ± 0.22 µm. Thus, NEG1 containing 7% snakehead fish extract and 3% lemon extract can facilitate wound healing by stimulating epidermal thickening and skin structure reconstruction more rapidly than NEG2.

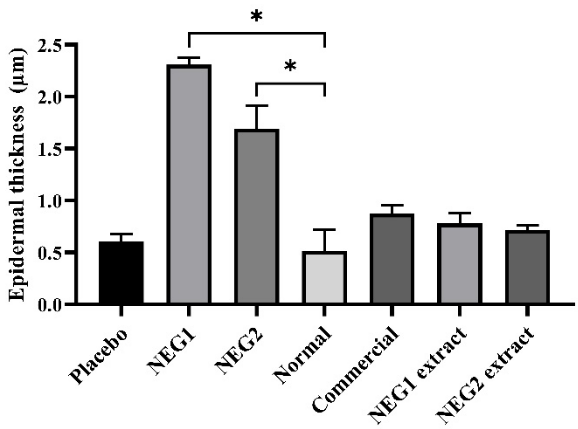


Figure 7. Diagram of the epidermal thickness on the 15th day of treatment. Symbol * expressed the significant difference ($p<0.05$) in comparison with normal group.

Based on the results of this study, it can be concluded that the nanoemulgel design has successfully optimized the activity of snakehead fish extract and lemon extract in wound healing. F1 nanoemulgel which contains 7% snakehead fish extract, and 3% lemon extract has better characteristics and effectiveness. The study revealed that F1 was histopathologically proven to stimulate post-cesarean section wound healing through re-epithelialization and reconstruction of skin tissue structure better than other formulas.

STATEMENT OF ETHICS

In vivo studies have been eligible for ethical eligibility based on the ethical eligibility letter issued by Moewardi Hospital, Surakarta, with letter number 1.220/VI/HREC/2023. In vivo testing procedures and animal welfare assurance have been approved by the head of the Experimental Animal Laboratory, Faculty of Medicine, Sebelas Maret University.

CONFLICT OF INTEREST STATEMENT

The authors report that there are no conflicts of interests.

AUTHOR CONTRIBUTIONS

AA, NAB, TASD designed the study. TASD, FAN worked on literature search. AA, NAB,TASD, FAN, SAR conducted the experimental work and collected the data. AA, NAB analyzed and interpreted the data. TASD, FAN, SAR wrote the draft of manuscript. All authors involved in revising the final manuscript. AA supervised the study and proofread the manuscript.

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