

The formulation and characterization of water-soluble snakehead fish (*Ophiocephalus striatus*) dry extract in nanoemulsion using permeation and *in vivo* study

Robert Tungadi¹ , Widysusanti Abdulkadir¹ , Munafri Tahir¹ 

¹State University of Gorontalo, Faculty of Sport and Health, Department of Pharmacy, Gorontalo, Indonesia

ORCID IDs of the authors: R.T. 0000-0003-2141-2402; W.A. 0000-0002-8975-134X; M.T. 0000-0002-9351-2843

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ABSTRACT

Background and Aims: The study was conducted to determine the optimal concentration of water-soluble snakehead fish dry extract (SFDE) in nanoemulsion and the amount of albumin required to penetrate the skin in order to accelerate the wound healing process.

Methods: The snakehead fish (SF) was extracted using an atomizer while the nanoemulsion basis was optimized using oleic acid, Tween 80, and propylene glycol. The developed SFDE in nanoemulsion was characterized based on droplet size, PDI, and zeta potential. The ability of the mixture to penetrate the snakeskin was tested using Franz diffusion cells. The effectiveness of the nanoemulsion was evaluated by dividing the rabbits used for experiment into 6 treatment groups including SFDE F1 0.25%, F2 0.5%, F3 1%, F4 SF 2% cream, F5 nanoemulsion basis, and F6 no treatment.

Results: The SFDE nanoemulsion produced a particle size of 147.5 nm with acceptable PDI (0.23) and zeta potential (+13.38 mV). The most effective SFDE to accelerate the healing of open wounds in rabbits was a concentration of 1%, which was found to have dried and closed the wound on the 3rd day.

Conclusion: The permeation study and the effectiveness test showed the 1% SFDE nanoemulsion is the best concentration in accelerating the wound healing process and ensuring the highest albumin penetration into the skin.

Keywords: Snakehead fish, nanoemulsion, albumin, wound, water-soluble, rabbit

INTRODUCTION

Snakehead fish (SF) (*Ophiocephalus striatus*) is an economically valuable fish widely used for processed products. According to Suprayitno (2003), it has a protein content estimated to be 25.1% compared to the 6.224% found in albumin and is higher than the values obtained from other animal sources used for patients with hypoalbuminemia (i.e. low albumin) and wounds. This is important because albumin has been discovered in medical science to have the ability of accelerating the recovery of broken body cell tissues due to surgery (Suprayitno, 2003; Ulandari, Kurniawan, & Putri, 2011).

Albumin is the largest type of protein in plasma with 60% content and also has the ability to synergize with zinc mineral needed for the development and formation of new cell tissues in wounds. Zinc has been reported to have the ability to functions as an antioxidant to protect cells, accelerate the wound healing process, and regulate expression of lymphocytes and proteins (Mustafa, Widodo, & Kristianto, 2012; Maryanto, 2004). Moreover, the chemical compounds of Snakehead fish dry extract (SFDE), including albumin and amino acids (glycine and lysine), have been discovered to be soluble in water based on chemical analysis tests from

Address for Correspondence:

Robert TUNGADI, e-mail: robert.tungadi@ung.ac.id

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LIPI conducted using spectrophotometry and HPLC methods. It is, therefore, the mix of these elements with nanoemulsion (NE) that is needed to obtain a homogenous system through the emulsification method (Zhang, Zhang, Fan, Liu, & Meng, 2019).

According to Tungadi, Susanty, Wicita, & Pido (2018), snake-head fish with 2% cream was found to have accelerated the healing process of rabbit skin's open wound in an *in vivo* study, but the cream was observed to be physically unstable after 3 months of storage. This was associated with the mixture of snakehead fish dry extract (SFDE) with macro emulsion, which causes effortless damage due to the strength of oil and water phase and storage temperature. A solution has, however, been reported which involves reducing the particle size of snake-head fish dry extract and stabilizer using nanoemulsion formulation through appropriate utilization of surface-active agents, co-surfactant, and oil (Tungadi, 2011; Devarajan, & Ravichandran, 2011). It is also possible to formulate the SFDE into the emulsion because it contains hydrophilic and hydrophobic compounds with the nanoemulsion discovered to be useful for transdermal drug delivery such as the penetration of active compounds due to stratum corneum deformability (Tungadi, Susanty, Wicita, & Pido, 2018).

Meanwhile, Tungadi, R. (2016) showed snakehead fish cream containing only 50% albumin has the ability to penetrate the skin membrane using penetrant enhancers such as propylene glycol. This, according to an *in vivo* study, has been reported to accelerate the healing of open wounds (treatment group) due to the increase in the rate of diffusing albumin into the stratum corneum. However, a low percentage of albumin is produced without the use of a penetrant enhancer (Tungadi & Hasan, 2016).

This shows a nanoemulsion system is suitable for the drug delivery through the skin due to its large surface area, which makes the penetration of active substances faster. It is also useful because its manufacturing process is very easy and efficient (Chuesiang, Siripatrawan, Sanguandeekul, McLandsborough, & McClements, 2018; Laxmi, Bhardwaj, Mehta, & Mehta, 2015) as observed in the formation of SFDE into dosage forms in Winda's research. This involved the optimization of nanoemulsion basis as a carrier for SF nanoemulsion preparation and later characterization by particle size, polydispersity index, and zeta potential with the results found to be 147.5 nm, 0.234, and +13.38 mV respectively (Tungadi, Moo, & Mozin, 2017). Therefore, this current study was conducted to determine the effectiveness of different concentrations of SFDE at 0.25, 0.5, and 1% in accelerating the healing of open wounds on rabbits dorsum and the amount of albumin required to penetrate their skin using the Franz diffusion cell.

MATERIALS AND METHODS

Materials

Snakehead fish dry extract was obtained from PT. Ismut Medical Pharmaceutical, Indonesia. The Rabbits were purchased from the animal market. The nanoemulsion basis (tween 80, propylene glycol, and oleic acid) was purchased from PT. Brataco Chemical. Other materials, such as propylparaben,

methylparaben, isopropyl myristate, lanolin, cetyl alcohol, paraffin liquid, and BHT were purchased from PT. Sentana Chemical. A UV-Vis Spectrophotometer (USA), Delsa™ Nano (UK), pH meter (Systronics model EQMK), sonicator (Specta Lab), hot air oven (Mettler), and the Franz diffusion cell (Intralab) were used.

Albino rabbits (2 kg) were obtained from the animal laboratory center of LIPI, Serpong, Indonesia. The experimental procedure was conducted according to the Institutional Animal Ethics Committee based on the recommendations of the Health Ethics Committee, The Faculty of Medicine, Hasanuddin University, Indonesia Government with registration No. UH08060042

The optimization and characterization of SFDE nanoemulsion basis

The nanoemulsion basis was optimized by comparing different concentrations of oil (oleic acid), co-surfactant (propylene glycol), and surfactant (Tween 80) using five formulas including F1 (1:2:4), F2 (1:3:4), F3 (1:3:5), F4 (1:3:6), and F5 (1:3:7). The Tween 80 and propylene glycol were mixed collectively using a magnetic stirrer for 30 minutes at 250 rpm. For the first mixture, the oleic acid was introduced during the stirring process. Water containing 0.25%, 0.5%, and 1% of SFDE was added drop by drop then other adjuvants, such as methylparabens and propylparabens (preservatives) as well as BHT (antioxidant) were added. After that, sonication of the mixtures at 20 KHz was performed for 10 minutes at 25°C to complete the process. The same procedure was performed for all the formulations with different concentrations of Tween 80, propylene glycol, and oleic acid. All formulations were characterized using a particle size analyzer to measure the size of droplets, zeta potential, and PDI.

Permeation study

In vitro permeation, conducted using Franz diffusion cell, has been described as a dependable technique to predict the transport of drugs in the skin (Zhu et al., 2009) and, for this study, an excised python skin (*Python reticulatus*) was used.

This process involved the separation of the skin from abundant fats and the elimination of connective tissue using a scalpel. The excised skin was washed with NaCl 0.9% and examined for integrity before it was hooked up on the diffusion cell with an effective diffusion area. Moreover, the stratum corneum facet was focused on the donor while the dermal layer was on the receiver compartment consisting of 47 ml phosphate buffer of pH 7.4 as the receptor fluid agitated at 100 rpm and maintained at 37±0.5°C during the experiments with 1 g of the nanoemulsion used in every diffusion cell. Approximately 2 ml of the samples were withdrawn for evaluation at 0, 30, 60, 90, 120, 150, 180, 210, and 240 min after the experiment has commenced and changed immediately with an equal volume of fresh diffusion medium (Tungadi et al., 2018).

Skin irritation study

Skin inflammation was evaluated using 12 healthy rabbits without any injuries or skin disorders. They were grouped into three with n=3 of albino male rabbits weighing 1.5-2 kg; positive control (2% w/w SFDE, commercial product), and negative control (nanoemulsion basis) also with n=3 on the 2 cm² dor-

sal facet of the shaven skin of the rabbits. The treatment was eliminated after 72 h to check for any symptoms of erythema and edema (Tungadi et al., 2018; Barot, Parejiya, Patel, Mehta, & Shelat, 2012; Lala, & Awari, 2014). Undesirable skin changes such as coloration and morphology were examined at 1h, 24 h, 48 h, and 72 h intervals. The reactions obtained were recorded and compared with a control group (n=3).

Effectiveness of the SFDE *in vivo* study

Preparation and grouping of test animals

The implementation stage started with the preparation of 12 male white rabbits randomly divided into 6 groups of treatments, each consisting of 2 rabbits, each of which were placed in individual cages and acclimated for 5 days. The Treatment Group contained SFDE varied at G1 0.25%, G2 0.5% and G3 1% of SFDE.

Testing of SFDE on test animals

The dorsal back of each test animal was shaved and cleaned with 70% alcohol after which they were locally anesthetized with 0.2 mL lidocaine and the wounds created by slicing off 4 cm² of skin and smearing the wounds with the SFDE treatments. The average change in length and the condition of the wounds were observed and documented every day for 10 days.

Measurement of the open wound area

The average length of the open wound was calculated using a ruler while pictures were also taken from day 0 to 10 to determine the healing process. The values measured in each day were converted to amount of contraction to determine the reduction effect of SFDE in different concentrations.

Statistical analysis

All the experimental measurements were recorded in triplicate and the final values were expressed as mean value±standard deviation (SD). The statistical evaluation of the permeation *in vitro* for the predetermined intervals was conducted using One-way ANOVA SPSS 16 with a degree of significance of P cost <0.05* and <0.01**.

RESULTS AND DISCUSSION

The formulation and optimization of nanoemulsion basis

There are several challenges to the application of nanoemulsion as a transdermal system to successfully deliver drugs via the skin (Kong, Chen, Kweon, & Park, 2011) and some of the important ones include the small particle-sized formulation and rheology properties. Therefore, it is necessary to understand the best formula to improve the introduction of snakehead fish dry extract (SFDE) into nanoemulsion using appropriate oil, surfactant, and co-surfactant (Tungadi et al., 2018).

The best optimization for nanoemulsion basis was found to be Formula 5 (F5) with oleic acid, tween eighty and propylene glycol (1:10) based on its viscosity, clarity, and stability as shown in Table 1.

Formula 5 was also observed to be physically stable by not segregating after being centrifuged at 3800 rpm for 5 hours while Formulas 1 to 4 produced a cloudy appearance and segregated. The stability was associated with the use of Tween 80 as a nonionic surfactant considering its excessive hydrophilic and lipophilic balance estimated at 15 which made it steady in an emulsion formulation with oil in water (Brandelero, Yamashita, & Grossmann, 2010).

Surfactant plays important roles in the nanoemulsion basis due to the fact it has a large surface area to decrease interfacial and surface tension, which further leads to its absorption in the interface phase. This means it has the ability to reduce the surface free energy by disintegrating a globule into smaller parts (Natalia, 2012). However, most surfactants are unable to decrease interfacial tension in the emulsion. Therefore, there is a need to add co-surfactant such a propylene glycol to improve the solubility of nonpolar agencies (Swarbrick, 2007), intensify the flexibility of surfactant film and fluidity of the emulsion phase to shield compounds from adverse environmental conditions, and enhance their balance (Madene, Jacquot, Scher, & Desobry, 2006; Kumar, Bishnoi, Shukla, & Jain, 2019).

Table 1. The optimization of nanoemulsion basis.

Materials	Formula %				
	F1 1:2:4	F2 1:3:4	F3 1:3:5	F4 1:3:6	F5 1:3:7
Oleic acid	5	5	5	5	5
Tween 80	18	20	23	25	27.5
Propylene glycol	12	15	17	20	22.5
Distilled water	100	100	100	100	100
Observation	cloudy	cloudy	cloudy	cloudy	clear
Stability tests:					
pH	6.5±0.3	6.2±0.5	6.0±0.7	5.8±0.2	5.5±0.1
Viscosity (cP)	385.6±1.3	267.8±2.5	200.3±2.1	187.5±3.2	178.2±1.4
Transmittance (%)	75.65±1.5	82.34±0.9	87.35±1.1	90.58±1.8	98.75±0.8

Characterization of snakehead fish nanoemulsion

Nanoemulsion systems can be used to deliver drugs through trans-mucosal and transdermal routes and this means they have the ability to effectively enhance bioavailability (Kumar et al., 2019; Rehman et al., 2017). The polydispersity index (PDI) of the SFDE produced good results in the three replications, 0.205, 0.215, and 0.284 respectively, and the 147.5 nm average droplet size shown in Table 2.

Table 2. The characterization of Snakehead Fish Nanoemulsion.

Sample	Particle size (nm)	Average of Size (nm)	Zeta potential (mV)	Polydispersity index (PDI)	Average of PDI
1% SFDE	111 ± 0.2	147.5 ± 0.53	+ 13.38	0.205 ± 0.1	0.23 ± 0.26
	233 ± 0.5			0.215 ± 0.2	
	98.6 ± 0.9			0.284 ± 0.5	

As shown in Table 2, the average size of the droplet of SFDE nanoemulsion was 147.5 nm showing that SFDE meets the criteria of nanostructures, which require a particle size range between 1 – 100 nm or 2 – 500 nm (Shah, Bhalodia, & Shelat, 2011). Meanwhile, the zeta potential value was +13.38 mV and this indicates it has a good degree of stability. This is associated with the standard that nanoparticles with values above or below ± 30 mV indicate a physically stable colloidal system due to their ability to ensure the magnitude of the charged particle prevents particle aggregation (Singh, & Lillard, 2014; Hadian, Sahari, & Moghimi, 2014). Meanwhile, smaller values have been reported to cause particles to aggregate and flocculate due to van der Waals attractive forces acting on them, thereby, leading to physical instability. Furthermore, the average polydispersity index was recorded to be 0.234 and this means SFDE has a uniform particle size and homogeneous dispersion because this value is below 0.25 (Winterhalter, & Lasic, 2013).

The solubility of active compounds is very important in drug formulation due to its ability to increase bioavailability through oral, topical, and parenteral formulations. SFDE contains water-soluble active compounds such as albumin and amino acids and water-insoluble ones such as polyunsaturated fatty acids, vitamins, and amino acids. This study made use of only the albumin and amino acid contents to ensure easy formulation into the nanoemulsion. Therefore, solubility is one of the important parameters to achieve the appropriate concentration of drug in systemic circulation and appropriate pharmacological response (Vemula, Lagishetty, & Lingala, 2010).

Permeation study of SFDE in nanoemulsion

Ex vivo permeation studies were also conducted using snake-skin as the membrane and the drugs from G1 (0.25% SFDE), G2 (0.5% SFDE), G3 (1% SFDE), and G4 (2% SF cream; commercial product) were found have produced $62.80 \pm 1.45\%$, $69.30 \pm 2.34\%$, $72.30 \pm 1.22\%$, and $50.80 \pm 0.50\%$ permeation, respectively in 4h as shown in Figure 1.

Figure 1 shows 2% SF cream had the lowest percentage of albumin permeation into the skin with approximately 50.80%

compared to all other concentrations and this is associated with the formulation of SFDE containing albumin into cream o/w to produce the big particle size in SF cream due to the macroemulsion. Its introduction to nanoemulsion produced a small particle size estimated to be 147.5 nm and water-soluble compounds with the ability to increase the loading capacity of albumin to penetrate the skin easily. This is consistent with the findings of previous research on the formulation of SFDE into

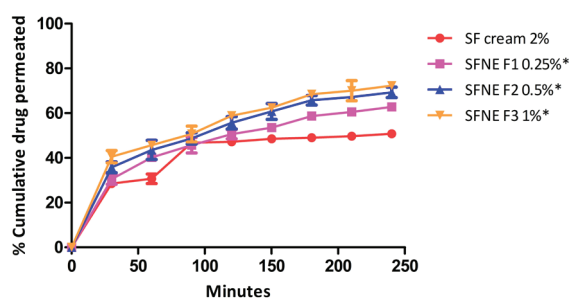


Figure 1. The amount of albumin penetrated into the skin in 4 h; * $P < 0.05$; One Way Anova Test.

liposome which showed solubility and particle size to be the most important factors to increase the loading capacity and bioavailability of drugs. SFDE into liposome was discovered to have a smaller particle size, 121 nm, compared to nanoemulsion and this led to the production of the highest entrapment efficiency of albumin recorded to be 85.75% (Tungadi, Abdulkadir, Ischak, & Rahim, 2019).

The biggest impediment to the transdermal drug transport is usually associated with the stratum corneum as observed in the 10-20 μm thick tissue layer which has a remarkably composed lipid/protein matrix structure (Ceve, 2004). According to Tungadi (2011), a study of SFDE cream containing penetrant enhancer such as propylene glycol is expected to accelerate the wound healing process through skin permeation, but the cumulative albumin penetration into rat skin membrane was recorded to be 50%. This study found SFDE nanoemulsion to have the ability to enhance the permeation of drug through the skin as observed from the cumulative percentage of SFDE permeation of F3 which was found to be the highest with $72.30 \pm 1.22\%$ using a snakeskin membrane while the positive control, SF cream 2%, had $50.80 \pm 0.50\%$. This, therefore, means nanoemulsion formulation acts as drug reservoirs in the transdermal delivery systems affecting the release of drugs from the inner to the outer phase and similarly to the skin (Tungadi

et al., 2018; Mou et al., 2008). These release mechanisms, however, rely on the composition of the network surfactant chains and the Crosslink density (Tungadi et al., 2018; Bernard, 2012). Moreover, the capacity of a drug to penetrate the skin and release the therapeutic agent effectively is affected by its affinity to diffuse out from the vehicle and permeate through the barrier (Tungadi et al., 2018; Alves, Scarrone, Santos, Pohlmann, & Guterren 2007).

In the current permeation study using Franz diffusion cell, *Phyton* snakeskin was used as a membrane to facilitate the penetration of the test substances compared to the use of extracts of stratum corneum isolated from the skins. This method was used in the study by Lin and colleagues (1992) and the permeability values in snakeskin (*Phyton molurus*) were found to be 2 to 4 times higher than in isolated stratum corneum for sodium diclofenate, theophylline, and benzoic acid (2 mg/mL or 0.2% in aqueous solution). The use of *phyton* snakeskin in studying SFDE nanoemulsion as a promoter of skin penetration for hydrophilic substances such as albumin required the consideration of the lower permeability coefficient (3.3 to 6.1 times) of these membranes for such compounds, thereby, causing an extension of the time needed for the experiments. Meanwhile, lipophilic compounds have been reported to have permeability coefficients close to those obtained from human skin membranes (0.9 to 1.8 times and 3.3 to 6.1 times) (Tungadi et al., 2019).

Skin irritation test of snakehead fish nanoemulsion

The results from the skin irritation study including erythema and edema on the rabbit skin after 1 h, 24 h, 48 h, and 72 h post-treatment of positive control, negative control, F1, F2, and F3 are represented in Table 3. The results showed no proof of inflammation, erythema, or edema; based on visible inspection after the application of all formulations of nanoemulsion on the rabbit skin during the three days of observation. This, therefore, means they were all non-sensitizing and safe for topical use.

Percentage of wound contraction on rabbit's skin

Based on observations, on the 3rd, 6th, and 10th-day, the open wound on the rabbit in group I (F1 0.25% SFDE) was found to have wound contraction percentages between 90% and 46% as shown in Figures 2-6 with the physical appearance marked by the presence of fibrin yarns protecting the open wound as presented in Figures 3-6. In group II (F2 0.5%), the reduction

was found to be 100% to 42% and was discovered to be drying in contrast to the observation made for group I. The results of group III (F3 1%) showed a substantial contraction from 100% to 25% compared to the negative control, which was observed to be faster. This change was characterized by the production of new granulation tissue on the side of the open wound and the fact that it was already dry on the third day. Furthermore, the positive control (F4) containing snakehead fish cream 2% had the change of wound contraction from 100% to 56%. The negative control F5 with nanoemulsion basis and F6 without treatment had the slowest healing process of approximately 15 days and a marked wound contraction exchange from 100% to 75-77% (Figures 2, 3-6).

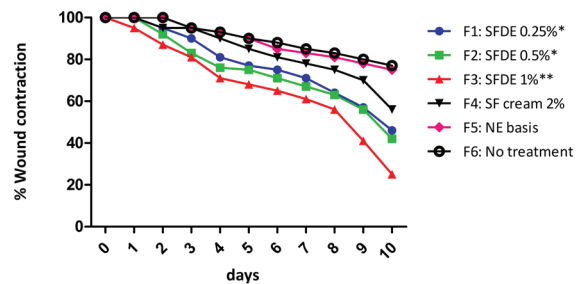


Figure 2. Percentage of wound contraction on rabbit's skin *P<0.05; **P<0.01; One Way Anova Test.

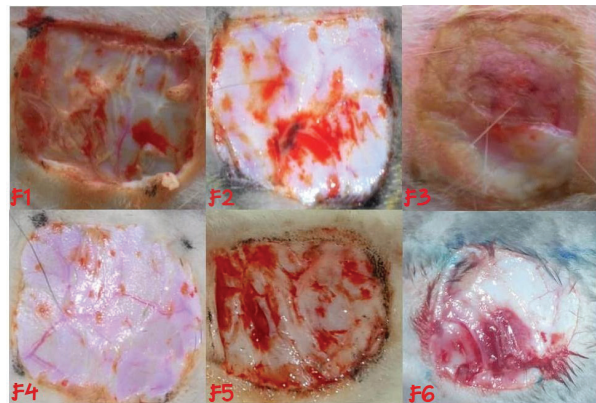


Figure 3. The observation of wound area on the first day, F1: NE 0.25% of SFDE; F2: NE 0.5%; F3: NE 1%; F4: 2% of SF cream; F5: NE basis; F6: no treatment.

	1 h		24 h		48 h		72 h	
	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema
G1 0.25%	0	0	0	0	0	0	0	0
G2 0.5%	0	0	0	0	0	0	0	0
G3 1%	0	0	0	0	0	0	0	0
Positive Control	0	0	0	0	0	0	0	0
Negative Control	0	0	0	0	0	0	0	0

Positive control: SF cream 2% (w/w); commercial product, negative control: nanoemulsion basis; Erythema scale: 0= none, 1=slight, 2= well-defined, 3= moderate, and 4= scar formation; Edema scale: 0= none, 1= slight, 2= well-defined, 3= moderate, and 4= severe

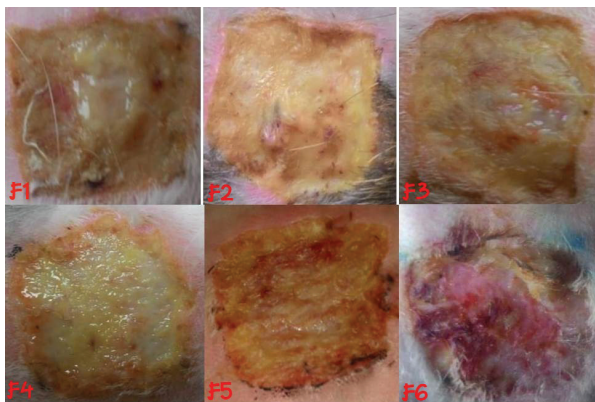


Figure 4. The observation of wound area on the third day, F1: NE 0.25% of SFDE; F2: NE 0.5%; F3: NE 1%; F4: 2% of SF cream; F5: NE basis; F6: no treatment.

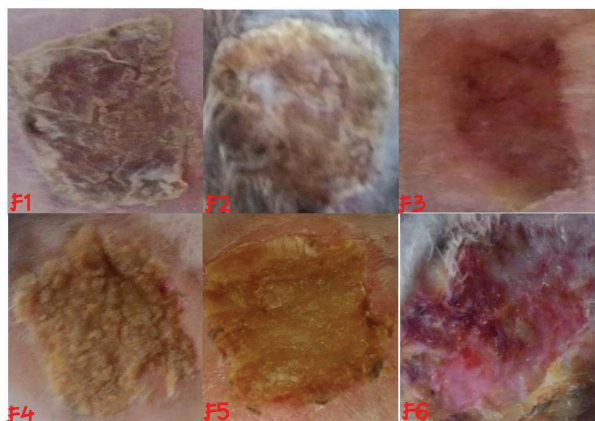


Figure 5. The observation of wound area on the sixth day, F1: NE 0.25% of SFDE; F2: NE 0.5%; F3: NE 1%; F4: 2% of SF cream; F5: NE basis; F6: no treatment.

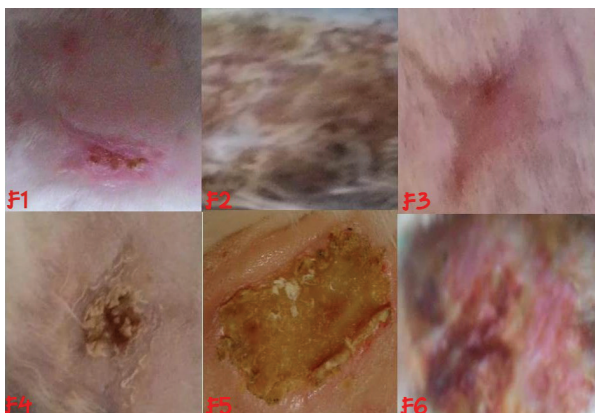


Figure 6. The observation of wound area on the ninth day, F1: NE 0.25% of SFDE; F2: NE 0.5%; F3: NE 1%; F4: 2% of SF cream; F5: NE basis; F6: no treatment.

The One-Way ANOVA analysis showed the P or Sig value was $0.022 < 0.05$ and 0.01 . This means there was a significant difference between the averages of open wound contraction for all treatment and control groups. However, observation data indicated NE 1% of SFDE had a faster wound area reduction compared to 0.25% and 0.5% nanoemulsion preparations and 2% SF cream.

Effectiveness of the SFDE *in vivo* study

F3 was found to be the best formula of SFDE nanoemulsion in this *in vivo* study functioning as a transdermal delivery system to ensure a controlled release of substances over a period and improve patient comfort during dosage preparation. Meanwhile, the small droplet size has been reported to have the ability to absorb albumin containing large molecules following the spontaneous size of the globule and surroundings (Lovelyn & Attama, 2011). The percentage of the albumin penetration and wound contraction of F3 were estimated at 72.30% and 25% on the 10th day. This was associated with the particle size and zeta potential of SFDE nanoemulsion because its small size of droplets increases the diffusion rate of albumin compared to micro or macro emulsion while the significant stability was due to the PDI and zeta potential.

The SFDE in nanoemulsion was able to accelerate the wound healing process due to the nutritional contents of snakehead fish including 0.003% Zn, 30.2% albumin, and 0.001% glycine (Mansyur, 2010) triggering the formation of Endothelial Progenitor Cells (EPC). The Zn plays a key role and has also been reported to be an important mineral in the structure and function of cell membranes by limiting the damage caused by free radicals during inflammation. Furthermore, it is also involved in the immune system, the defense of the skin, and the regulation of genes in lymphocytes (Tungadi et al., 2019; gawhirunpat, Panomsuk, Opanasopit, Rojanata, & Hatanaka, 2006; Tungadi, & Wicita, 2020).

CONCLUSION

It is possible to formulate water-soluble snakehead fish dry extract into nanoemulsion with small particles to increase the loading capacity of albumin in penetrating the skin. The permeation study and the effectiveness test showed the 1% SFDE in nanoemulsion is the best concentration compared to others in accelerating the wound healing process and ensuring the highest albumin penetration into the skin.

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