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Research Article

Transdermal Delivery of Snakehead Fish (*Ophiocephalus striatus*) Nanoemulgel Containing Hydrophobic Powder for Burn Wound

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ABSTRACT

Background: The aim of the present study was to characterize and evaluate the nanoemulgel (NEG) of snakehead fish powder (SFP), as a transdermal delivery system for poorly water soluble drug, in order to conquer the inconveniences related to its oral conveyance.

Methods: Diverse nanoemulsion components (oil, surfactant, and co-surfactant) were chosen based on solvency and emulsification capacity. SFP loaded nanoemulsion which tested by stress-stability testing was carried out for all formulations and those that passed these tests were characterized for mean droplet size, polydispersity index (PDI), zeta potential, pH, viscosity, and transmittance. After that, this was continued by permeation studies using snake skin in vitro and rabbit skin in vivo studies i.e. skin irritation study and the effectiveness test.

Results: Mean droplet size and zeta potential of the optimized nanoemulsion (NE4) were found to be 98.6 ± 0.93 nm (polydispersity index, PDI = 0.1 ± 0.20) and -57.5 ± 0.3 mV respectively. Optimized nanoemulsion was converted into nanoemulgel with 1.5% w/v of gelling agent (HPMC) and evaluated for pH, viscosity, spreadability, and extrudability measurement. Ex vivo transdermal permeation value for SFP through snake skin as membrane from NEG_1, NEG_2, NEG_3 and marketed SFP cream showed results of 55.65 ± 0.93%, 56.14 ± 0.70%, 66.75 ± 1.03% and 49.80 ± 3.42% respectively in 3 hours. Moreover, all the treatment group did not show skin irritation of each group. The effect of burn wound healing of NEG3 showed a significant ($P<0.05$) on the measurement of wound area compared to marketed cream.

Conclusion: The novel NEG of SFP was successfully formulated for transdermal application based on the results of evaluations and stability tests on accelerating burn wound healing.

Introduction

In Indonesia, snakehead fish (*Ophiocephalus striatus*) are one of freshwater fish which used people as foodstuff for accelerating wound healing after post-operation. Some researchers have done some research about snakehead fish in other dosage forms such as tablet, capsule and cream. At the moment, pharmaceutical products of snakehead fish are developed by researchers in the form of cream preparations by Tungadi (2016), capsule preparations by Tawali, et al. (2012) and suspension preparations by Lawang (2013).[1-3]

Suprayitno (2003) stated that the giving of albumin therapy with snakehead water extract orally can assist wound healing process faster.[4] Meanwhile, according to Tungadi (2016), snakehead fish powder had been formulated into a cream for accelerating wound healing of post-operation *in vitro and in vivo*. However, the snakehead fish cream was physically unstable, so that the emulsion system was easily broken by adding energy of oil, water phase and storage temperature. Besides that, the giving of snakehead fish, meat or water extract are not so favored because they have an unpleasant odor and has a high fat content causing the process of degradation quickly and rancid smell. Therefore, this problem can be solved by formulation development in nanoemulgel as transdermal delivery for burn wound healing.

Nanoemulsion is an emulsion system having the droplet size in nanometer scale in which oil or water droplets (20-200 nm) are finely dispersed in the opposite phase with the help of a suitable surfactant to stabilize the system.[5] The ascendancies associated with transdermal use of nanoemulsion are as enhanced drug solubility, good thermodynamic stability and enhancing effect on transdermal ability.[6-7] The aptness of nanoemulsion is to increase the concentration gradient and thermodynamic activity of its components makes the system expedient for transdermal delivery.[8] Unlike microemulsions, which require a high surfactant concentration, my animations are formulated with a reasonable surfactant concentration for the efficient topical delivery of API owing to their small

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droplet size, large surface area and low surface tension. They allow rapid penetration of lipophilic actives and thus improving efficacy and minimizing side effects by reducing the dose. Nominations may be used as a substitute for other less-stable lipid nanocarriers (for example, liposomes and vesicles) to improve transdermal permeation of many drugs over the conventional topical formulations. [9-12] Due to improved physical stability, and their non-toxic and non-irritant nature, they can be employed in topical drug delivery systems, providing greater absorption of solubilized lipophilic drugs.

Meanwhile, the low viscosity for nanoemulsion obliges its requisition in transdermal delivery because of awkward use. Biocompatible gels having feeble interaction with surfactants bring as of now being investigated to change those rheological conduct technique of nanoemulsion.[13-14] Variant gel matrices such as Carbomer 940, xanthan gum, methylcellulose and carrageenan have been exploited to increase the viscosity of nanoemulsion for transdermal delivery.[15] Thus the incorporation of nanoemulsion into a gel matrix can result in nanoemulgel which may be more relevant for transdermal application as compared to nanoemulsion. This means that it does not need a penetrant enhancer to accelerate the diffusion rate of active compounds into membrane cells.

Snakehead fish powder (SFP) contains a high protein, total such as albumin, amino acids and unsaturated fatty acids having a function to accelerate wound healing with formation of new tissues.[16] Besides that, albumin of snakehead fish was utilized to accelerate burn wound healing, increase the amount of blood protein, improve fracture and prevent lung infection.[3] It means that SFP has hydrophobic and hydrophilic compounds and macromolecule size about 20 µm which are difficult to penetrate into membrane cell. Moreover, it is also associated with oral side effects, including doses, stench, and nausea when administered by oral route. The promising method to diminish its adverse effects is to deliver the SFP powder via the skin.

According to Tungadi (2018) stated that snakehead fish cream (negative control) was difficult to penetrate the stratum corneum using rabbits in vivo, which could be seen open wound longer recovery than using penetrant enhancer such as propylene glycol (treatment group). This means that propylene glycol can accelerate the diffusion rate of albumin into stratum corneum, which the amount of albumin around 49% compared to without penetrate enhancer about 5-7%. Therefore, SFP was formulated into nanoemulgel using the best comparison of surfactant, co-surfactant and oil appropriately. The characterization of snakehead fish powder nanoemulsion has important roles in showing stability measurements such as particle size, zeta potential, and poly-dispersion index by particle size analyzer.[17]

The present study aims to characterize and evaluate of snakehead fish powder nanoemulgel as topical drug delivery by permeation study in vitro and the components of nanoemulsion system are expected to act themselves as permeation enhancer and a gel matrix as a cooling agent for burn wound healing in vivo study.

Materials and Methods

Materials

Snakehead fish powder of pharmaceutical grade was gained by PT. Royal Medical, Pharmaceutical, Indonesia, and was certified containing protein, 85.6%, albumin 30.2%, omega-3 2.03%, omega-6 2.11% and omega-9 0.92% and polyunsaturated total 5.1% respectively. The gelling agent, HPMC 22.000, coconut oil, olive oil, and cremophor EL were purchased from PT. Brataco Chemical (Bandung, Indonesia). Labrafac PG, triacetin, and Squalene were kindly supplied by Sigma-Aldrich (Singapore). Isopropyl myristate, oleic acid, castor oil, tween 80, tween 60, PEG 200 and 400, and propylene glycol were bought from PT. Intraco Chemical (Makassar, Indonesia). DMDM Hydantoin and BHT were purchased from PT. Sentana Chemical (Makassar, Indonesia).

The experimental protocols were approved by the Institutional Animal Ethics Committee as per guidelines of the health ethics committee, The Faculty of Medicine, Hasanuddin University, Indonesia Government with registration number UH 16060042.

Formulation Development of Nanoemulsion

Screening of Components

The criterion for screening of components was the solubility of SFP in different oils, surfactants and co-surfactants (Table 1). An excess amount of drug was added to 5 ml of the selected oil, surfactant and co-surfactant in a glass vial, sealed and stored upright. The samples were vortexed and maintained at 37 ± 1°C for 72 h in a shaking incubator (Memmert, Germany) to facilitate solubilization. To remove the undissolved drug, samples were centrifuged (Hettich EBA 200, Germany) at 3000 RPM for 15 min. The supernatant was filtered through a 0.22-µm nylon syringe filter. The solubility of the SFP was determined by Spectrophotometry UV-Vis (Perkin Elmer, USA).[18]

Preparation of Drug-Loaded Nanoemulgel

Aqueous titration method, followed by low-pressure homogenization (LPH), was used for the preparation of nanoemulsions. Construction of a pseudo-ternary phase diagram was done for the determination of concentration range of different components. Surfactant and co-surfactant (S:CoS) were mixed in different weight ratio (3:4, 3.5:4.5, 4:5, 4.5:5.5, 5:6 and 5.5:6.5), either in increasing concentration ratios of surfactant or co-surfactant, with respect to each other.[19] Under moderate agitation on a magnetic stirrer (Heidolph, Germany) for 30 min 250 RPM, different weight ratio of oil and Smix (S:CoS) were diluted dropwise with the aqueous phase to form the crude nanoemulsion to get a desirable mean droplet size of less than 200 nm. Later, the product was visually assessed on the basis of clarity, transparency and flow ability, and then finally categorized as

nanoemulsions. The drug-loaded nanoemulsions were prepared by dissolving 0.125%, 0.25% and 0.5% (w/w) of SFP in the mixture of oil and Smix. The developed formulations were then subjected to different stress-stability tests.

Stress-Stability Studies

Heating-Cooling Cycle

The formulations were subjected to heating-cooling cycles (n=3) over a temperature range of 4-40ºC, stored at each temperature condition for 48 h and observed for any physical instability in the form of phase separation, flocculation or precipitation.[19,20]

Centrifugation

The selected nanoemulsions formulations were centrifuged at 3500 RPM for 30 min to see phase separation which related to homogeneity of nanoemulsions.[19,20]

Freeze-Thaw Cycle

Nanoemulsions formulations were kept between temperature -21ºC and +25ºC, for three cycles for a duration of 48 h, and examined for changes in homogeneity of nanoemulsions[19,20]

Characterization of Nanoemulgel

Droplet Size

For determining the behavior of nanoemulsions, mean droplet size is very important. It was determined by using Delsa™ Nano Z (Beckman Coulter, UK) based on the principle of photon correlation spectroscopy, which analyses fluctuation in light scattering due to Brownian motion of particles. Light scattering was monitored at 25ºC at a scattering angle of 90º. The animation 1 ml was transferred to a disposable polystyrene Corbett with the help of a micropipette, and the mean droplet size was determined in triplicate.

Zeta Potential

Zeta potential is the electric potential which exists on the hydrodynamic plane of shear of a particle. It was measured by applying an electric field across the dispersion. Nanoemulsions were placed in clear disposable zeta cells, and zeta potential, which indicates the surface charge of the developed nanoemulsions, was measured by zetasizer (Delsa™ Nano instruments, UK) at 25ºC in triplicate.

pH, Viscosity and Transmittance

The pH values for nanoemulsions were determined at 25ºC by a calibrated pH meter (Systronics model EQMK, India). The viscosity of nanoemulsions was measured using viscometer Brookfield (DV-E Model, USA), equipped with a cone-plate type measuring system. The rheological studies were carried out at variable shear rates ranging from 1 to 100 s^{-1}. The transmittance was observed by using Spectrophotometry UV-Vis (Perkin Elmer, USA) at 630 nm. One milliliter of nanoemulsion formulation was taken in a test tube and water dilution was analysed at 630 nm in triplicate.

Stability Studies of Nanoemulgel

Nanoemulsions were assessed at accelerated conditions of storage with varying temperature and humidity, as per ICH guidelines.[21] Nanoemulsions were placed in 5 ml glass vial, sealed and stored upright. Physical and chemical stabilities of nanoemulsions were evaluated for 3 months by storing them at three different temperature and humidity conditions (25±2ºC/60 ± 5% relative humidity (RH), 40±2ºC/65 ± 5% RH and 60 ± 2ºC/75 ± 5% RH) and then characterized at specific time intervals for various parameters.[22] The nanoemulsions were also visually observed for any signs of turbidity, phase separation, coalescence, etc.

HPMC was made in different concentrations i.e.1.5 %, 2.0% and 2.5% w/v respectively. Each concentration of HPMC was weighed according to concentrations of each formula. After that, HPMC was dispersed into water and allowed to stand for overnight, then stirred 500 RPM for 3 minutes to form a clear gel with appropriate viscosity and then the optimized nanoemulsion was incorporated into the gel base. The prepared nanoemulgel formulations were inspected visually for their color, appearance and consistency.[23]

Prepared nanoemulgels (≈5 g) were packed in aluminium collapsible tubes and kept for stability studies at 25±2°C/60 ± 5% RH, 40±2°C/65 ± 5% RH and 60±2°C/75 ± 5% RH for a period of 3 months, as per ICH guidelines.[24,21,25-27] At an interval of 15 days, samples were withdrawn and evaluated for physical appearance, pH, viscosity, spreadability, and extrudability.

Evaluation of Nanoemulgel

Spreadability Study

Spreadability was determined by utilizing an apparatus suggested by Mutimer et al.[24] There was a wooden block and a pulley attached to it at one end. On the basis of 'slip' and 'drag' characteristics of nanoemulgel, spreadability measurement was done. An excess of nanoemulgel (≈2 g) was placed between two uniform slides placed on the block, where one glass slide was fixed and another was attached to a pulley. On the top of the two slides, a 1 kg weight was placed for 5 min to provide a uniform film of the nanoemulgel between the slides. The time taken by the upper slide to move on the application of weight to it through the pulley was noted, and spreadability was calculated by using the following formula, in triplicate:

$S = M \times L/T$

S: Spreadability, M: Weight applied to upper slide, L: Length of the glass slide, T: Time taken to separate the slides completely from each other

Extrudability Study

It is a test to measure the force required to extrude the gel from the tube. On the application of weight, the amount of gel extruded from the aluminium tube was determined. The nanoemulgel extruded should be at least 0.5 cm

ribbon in 10 s.[25] The higher the quantity of gel extruded, the better is the extrudability. The extrudability of each formulation was measured, in triplicate, and calculated by using the formula:

$E = M/A$

E: Extrudability, M: Applied weight to extrude gel from tube, A: Area

Transdermal Permeation Study

The in vitro permeation studies were carried out using Franz diffusion cell, which is a reliable method for prediction of drug transport across the skin.[28] These studies were conducted employing excised skin of python (*Python reticulatus*).

The python skin was separated, excess fat and connective tissue were removed using a scalpel. The excised skin washed with normal saline, examined for integrity and subsequently used. The skin was mounted on diffusion cell assembly with an effective diffusion area of 9.07 cm^2, where the stratum corneum side was facing the donor compartment and dermal side was facing the receiver compartment. The receptor compartment consisted of 47 ml phosphate buffer of pH 7.4 as receptor fluid agitated at 100 RPM and maintained at 37±0.5ºC throughout the experiments. 1 g of the nanoemulgel was used in each diffusion cell. 2 ml samples were withdrawn for analysis at 0, 60, 120, 180 min after commencement of the experiment and replaced immediately with an equal volume of fresh diffusion medium.

Skin Irritation Study

The experimental protocols were approved by the Institutional Animal Ethics Committee as per guidelines of the health ethics committee, The Faculty of Medicine, Hasanuddin University, Indonesia Government with registration number UH 16060042.

Skin irritation test using healthy 10 rabbits, there were no injuries or skin disorders. Three groups (n=3) of albino male rabbits (1.5-2 kg) were used in the study. Positive control (2% w/w SFP), nanoemulgel and negative control (gel basis) were applied (n=3) on the dorsal side (2 cm^2) of properly shaven skin of rabbits. The formulation was removed after 72 h and examined for any signs of erythema and edema.[29,30] Undesirable skin changes, i.e. change in color and change in skin morphology, were visually checked in for periods of 1h, 24 h, 48 h, and 72 h. The resulting reactions were compared and scored against a control group (n=3).

The Effectiveness Test in vivo

The best results of formula optimization and penetration test were continued with the effectiveness test of nanoemulgel using rabbit skin. All the treatment groups utilized 10 rabbits which each group consisted of 2 rabbits. The rabbits were divided into 5 groups, i.e. group I was applying nanoemulgel containing 0.125% of SFP group II: 0.25% of SFP, group III: 0.5% w/v of SFP, group IV: snakehead fish cream (marketed cream) and group V: nanoemulgel basis. All rabbits were shaved the hair on dorsal side using surgical scissors and sterile scalpel. The rabbit dorsal were anesthetized with Lidocain® injection, then each rabbit was wounded at the left dorsal by induction of hot metal having diameter 2.5 cm for 5 seconds to get burned wound. After that, the burn wounds were applied by snakehead fish nanoemulgel in accordance with treatment and control group then all groups were made observations for 9 days, including measurement of wound diameter and taking pictures of wounds every 3 days.

Statistical Analysis

All experimental measurements were performed in triplicate. Results value was expressed as mean value ± standard deviation (SD). Statistical analysis of permeation in vitro among predetermined intervals between formulation and the burn wound measurement and also the evaluation of snakehead fish nanoemulgel was performed by using One Way Anova SPSS 16. The level of significance was taken at P value < 0.05.

Results

Formulation Development of Nanoemulsion (NE)

All excipients for the formulation were selected from among the "Generally-Recognized-as-Safe" (GRAS) category (Table 1). Olive oil was selected as the oil phase due to the highest solubility of the snakehead fish powder (6.5 ± 0.25 mg/ml) as compared to other experimental oils. Tween 80 and Propylene glycol were utilized as the surfactant and co-surfactants respectively, since they showed the maximum drug solubility, i.e. 6.2 ± 0.40 mg/ml and 5.8 ± 0.17 mg/ml (Table 1). There were a lot of ratio Smix but only four ratios of six were the best ratio, i.e. 4:5, 4.5:5.5, 5:6, and 5.5:6.5, as shown in Figure. 1. Oil phase and Smix then mixed in the weight ratio of 1:12 to 12:1 of oil and Smix were chosen, so that maximum ratio could be covered in the study. About 0.125% (w/w) SFP was loaded in the formulation by solubilizing the SFP in oil and Smix phase. The aqueous phase was added in the range of 5%-95% (v/v) of the total volume of nanoemulsion at 5% intervals to form the crude nanoemulsions using magnetic stirrer 250 RPM for 30 min to gain more uniform-sized droplets. The nanoemulsion formulations were subjected to stress-stability testing, including heating-cooling cycle, centrifugation and freeze-thaw cycles (Table 2), and showed stability, were further characterized. We selected one nanoemulsion (NE1, NE2, NE3, and NE4) which was the best ratio of Smix. Through a titration method, the mean droplet sizes for the formulations NE1, NE2, NE3 and NE4 were found to be 534.6±2.5, 430.8±3.81, 378.3±2.8 and 127.1±2.6 nm respectively (Table 3, C0). After running 3 cycles of low pressure homogenization at 500 RPM, mean droplet size was effectively reduced to 204.3±2.2, 175.2±2.6, 140.6±1.8 and 98.6±0.93 nm for NE1, NE2, NE3 and NE4, respectively, having a polydispersity index (PDI) values of 0.3±0.08, 0.2±0.01, 0.15±0.20 and 0.10±0.2, respectively.

Table 1. Solubility profile of snakehead fish powder in different excipients (oils, surfactants and co-surfactants).

	Name of excipient	Solubility (mg/mL)
Oils		
	Coconut oil	2.5 ± 0.11
	Labrafac PG	4.3 ± 0.17
	Olive oil	6.5 ± 0.25
	Isopropyl myristate	5.0 ± 0.65
	Oleic acid	6.0 ± 0.15
	Castor oil	3.2 ± 0.25
	Triacetin	2.5 ± 0.15
	Squalene	4.1 ± 0.28
Surfactants		
	Tween 80	6.2 ± 0.40
	Tween 60	5.2 ± 0.29
	Cremophor EL	5.0 ± 0.32
Co-surfactants		
	Polyethylene glycol 200 (PEG 200)	4.0 ± 0.15
	Polyethylene glycol 400 (PEG 400)	5.2 ± 0.12
	Propylene glycol	5.8 ± 0.17

All values are reported as mean ± S.D (n=3).

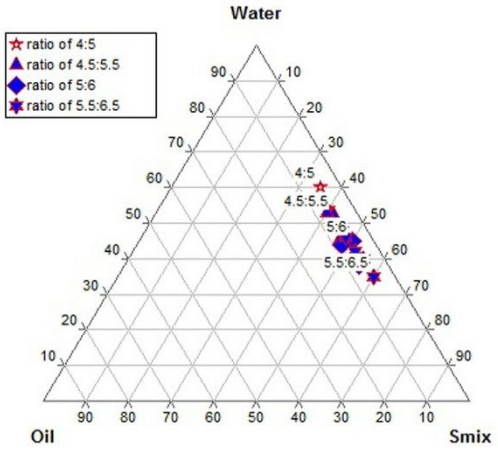

Figure 1. Pseudo-tertinary phase diagrams of the nanoemulsion formulations composed of oil phase (olive oil) and Smix (Tween 80 and propylene glycol).

Characterization of Nanoemulgel (NEG)

Based on the best result of nanoemulsion optimization, the smallest size and PDI, was NE4 is having the average of zeta potential value after stability study around -59.5±0.43 MV. Meanwhile, the pH values of nanoemulsion formulation before the stability test were found to be in the range of 5.0±0.35 to 6.0±1.14 (Table 4) and after the stability study, the average of nanoemulsion pH was 5.5±0.45 to 5.8±0.26 (Table 5). The viscosities of nanoemulsion formulation, measured at a shear rate of 100 s^{-1},[31] were found the range between 135.8±0.36 and 168.2±0.21 cP.

Transmission test of formulations was performed and transmittance values of all formulations were found to be in the range of 97% - 99% (Table 6). This showed that all formulations were clear and transparent. After that, the evaluation of nanoemulsion formulations, optimized formulation (NE4) was selected for the stability studies and further formulated into a nanoemulgel.

Stability Studies of Nanoemulgel

The stability studies showed that during the storage period of 3 months at 25±2ºC/60±5% RH, 40±2ºC/65±5% RH and 60±2ºC/75±5% RH, optimized nanoemulsion (NE4) described very negligible changes in mean droplet size, zeta potential, PDI, pH, viscosity, and transmittance (Table 5). The results gave no phase separation and flocculation, proving its stable nature.

Table 2. Stress stability screening of various nanoemulsion formulations.

Smix ratio	Percentage of components (v/v)			Observations		Inference	
	Oil	Smix	water	HC	Cent	FT	
4:5	5	35	60	x	x	x	failed
	6	50	44	√	√	√	passed
	6	40	54	√	x	-	failed
	7	44	49	√	x	√	failed
4.5:5.5	6	42	52	x	√	x	failed
	7	40	53	x	√	-	failed
	7	45	48	√	√	√	passed
	8	47	45	√	√	√	passed
5:6	5	50	45	√	-	√	failed
	6	45	49	x	√	√	failed
	7	55	38	√	√	√	passed
	8	48	44	√	√	√	passed
5.5:6.5	5	60	35	√	√	√	passed
	5	55	40	√	√	√	passed
	6	52	42	√	x	√	failed
	6	48	46	√	√	x	failed

HC: heating-cooling cycle, Cent: centrifugation, FT: Freeze-thaw cycle, (√) no phase separation, (x) phase separation, (-) not done.

Table 3. Effect of low-pressure homogenization process variables on mean droplet size (DS) and oolydispersity index (PDI) of various nanoemulsion formulations.

No. of cycles	Formulations							
	NE1		NE2		NE3		NE4	
	DS±SD (nm)	PDI±SD	DS±SD (nm)	PDI±SD	DS±SD (nm)	PDI±SD	DS±SD (nm)	PDI±SD
C0 (Crude nanoemulsion)	534.6±2.5	0.12±0.02	430.8±3.8	0.2±0.01	378.3±2.8	0.3±0.09	127.1±2.6	0.2±0.17
C1	365.4±2.0	0.09±0.04	306.4±2.7	0.1±0.02	286.5±2.5	0.1±0.12	87.5±0.51	0.2±0.23
C2	287.2±3.5	0.15±0.15	246.9±3.5	0.3±0.02	187.2±2.0	0.2±0.21	96.3±0.22	0.2±0.12
C3	204.3±2.2	0.37±0.08	175.2±2.6	0.2±0.01	140.6±1.8	0.1±0.20	98.6±0.93	0.1±0.20

All values are reported as mean ± SD (n=3)
C0: pre-homogenized nanoemulsion (crude nanoemulsion), PDI: polydispersity index, SD: Standard deviation.

Table 4. Determination of pH, viscosity and transmittance of various nanoemulsion formulations.

Formulation Code	Smix ratio	pH	V(cP)	t (%)
NE1	4:5	5.5 ± 0.23	154.3 ± 0.38	98.76 ± 0.05
NE2	4.5:5.5	5.0 ± 0.35	168.2 ± 0.21	97.78 ± 0.06
NE3	5:6	6.0 ± 0.18	135.8 ± 0.36	98.46 ± 0.03
NE4	5.5:6.5	6.0 ± 1.14	136.7 ± 0.24	99.87 ± 0.08

All values are reported as mean ± SD (n=3)
V: viscosity, cP: centipoise, t: transmittance

Table 5. Stability study of optimized nanoemulsion (NE4).

T (°C)/RH (%)	Time (days)	Mean (nm) droplet size	Zeta (mV) potentia	PDI	pH	V(cP)	t (%)
25±2/60±5	30	100.3±2.8	-58.3±0.6	0.24±0.06	6.1±0.22	140.7±1.34	98.28±0.16
	60	125.8±3.2	-45.9±0.7	0.35±0.05	6.0±0.32	148.5±1.85	97.35±0.28
	90	110.5±2.2	-48.3±0.5	0.38±0.04	6.3±0.18	150.2±1.56	98.24±0.34
40±2/65±5	30	120.7±3.5	-60.5±0.4	0.23±0.05	5.8±0.24	138.5±2.35	98.20±0.37
	60	95.32±5.2	-55.6±0.3	0.37±0.03	5.7±0.18	130.5±1.54	97.58±0.28
	90	87.48±3.7	-60.4±0.2	0.28±0.03	5.9±0.27	140.8±1.52	98.24±0.27
60±2/75±5	30	68.44±5.2	-57.5±0.3	0.27±0.02	5.8±0.26	137.6±1.87	99.25±0.23
	60	72.57±3.4	-60.3±0.4	0.22±0.02	5.6±0.20	138.2±2.12	98.65±0.26
	90	99.90±2.0	-60.7±0.6	0.30±0.03	5.5±0.45	137.8±2.03	97.34±0.43

All values are reported as mean ± SD (n=3)
T: temperature, PDI: poly-dispersity index, V: viscosity, cP: centripoise, t: transmittance

Table 6. Composition and evaluation parameters of nanoemulgels.

Composition	$NEG_{1.5}$	$NEG_{2.0}$	$NEG_{2.5}$
Snakehead fish powder (%w/w)	0.125	0.125	0.125
HPMC 22000 (%w/v)	1.5	2.0	2.5
Tween 80 (%w/w)	32.5	32.5	32.5
Propylene glycol (%w/w)	27.5	27.5	27.5
Olive oil (%w/w)	5	5	5
BHT (%w/v)	0.1	0.1	0.1
DMD Hydantoin (%w/v)	0.1	0.1	0.1
Water	q.s.	q.s.	q.s.
Evaluation parameters			
pH	6.0 ± 0.27	6.3 ± 0.35	6.2 ± 0.22
Viscosity (cP)	210 ± 3.34	458 ± 5.20	765 ± 4.53
Spreadability (g cm/s)	5.8 ± 0.15	3.9 ± 0.25	2.8 ± 0.56
Extrudability (g/cm^2)	1.4 ± 0.44	1.6 ± 0.65	2.0 ± 0.78

All values are reported as mean ± SD (n=3)
q.s.: quantity sufficient, cP: centipoise, g: gram, cm: centimeter, s: second

At the end of three months, the drug content was assayed and was found to be more 90% of the initial drug added, showing the chemical stability of the system during the storage period.

Nanoemulgel was prepared by adding NE4 into overnight-soaked gelling agent (HPMC 22000) at 1.5% (w/v) $NEG_{1.5}$, 2.0% (w/v) $NEG_{2.0}$ and 2.5% (w/v) $NEG_{2.5}$ with continuous stirring at 500 RPM.[32-34] The final formulation also contained BHT as antioxidant and DMDM Hydantoin as preservative.

Evaluation of Nanoemulgel

The pH values for $NEG_{1.5}$, $NEG_{2.0}$ and $NEG_{2.5}$ were found to be in the range of 6.0±0.27 to 6.3±0.35 (Table 6). Viscosities for $NEG_{1.5}$, $NEG_{2.0}$ and $NEG_{2.5}$ were found to be 210±3.34, 458±5.20 and 765±4.53 cP, respectively. Furthermore, spreadability values for $NEG_{1.5}$, $NEG_{2.0}$ and $NEG_{2.5}$ were found to be about 5.8±0.15, 3.9±0.25 and 2.8±0.56 cm, respectively (Table 6). Extrudabilities of $NEG_{1.5}$, $NEG_{2.0}$ and $NEG_{2.5}$ were found to be 1.4±0.44, 1.6±0.65 and 2.0±0.78 cm, respectively, in 10s on

applying a weight of 400 g (Table 6). In assessing spreadability and extrudability values of nanoemulgel formulation, $NEG_{1.5}$ was found to be 0.76±0.05% (w/w). $NEG_{1.5}$ was found to be stable during three months storage period at different conditions of temperature and humidity. There was no change in physical appearance, pH and viscosity (data not shown). During this storage period, the drug content was assayed and was found to be more 90% of the initial drug loaded (0.76±0.05% (w/w) of gel base), confirming the chemical stability of the system.

Transdermal Permeation Study

Ex vivo permeation studies were carried out using snake skin as membrane. Permeation of drug from NEG1 (0.125% SFP), NEG2 (0.25% SFP), NEG3 (0.5% SFP) and marketed cream (2% SFP) were found to be 54.92±1.06%, 56.00±0.83%, 65.02±1.08% and 49.34±2.57%, respectively, in 3h (Figure 2).

Skin Irritation Study

The scores of skin irritation study (erythema and edema) on rabbit skin after 1 h, 24 h, 48 h and 72 h post treatment of positive control, negative control, NEG1, NEG2 and NEG3 are represented in Table 7.

The Effectiveness Test of SFP Nanoemulgel In Vivo

Based on observations can be seen that on the 3rd, 6th, 9th day, burn wound on the rabbit treatment of group I which, given $NEG_1$0.125% SFP showed a wound area change from 2.2 to 1.25 cm^2 (Table 8) and physical appearance of the wound which marked by the presence of fibrin yarns covering the burn wound. Meanwhile, group II, which given NEG_2 0.25% showed wound area changes from 2.3 to 0.85 cm^2 and gave the reduction of wound area and the wound condition becoming dry compared to group I.

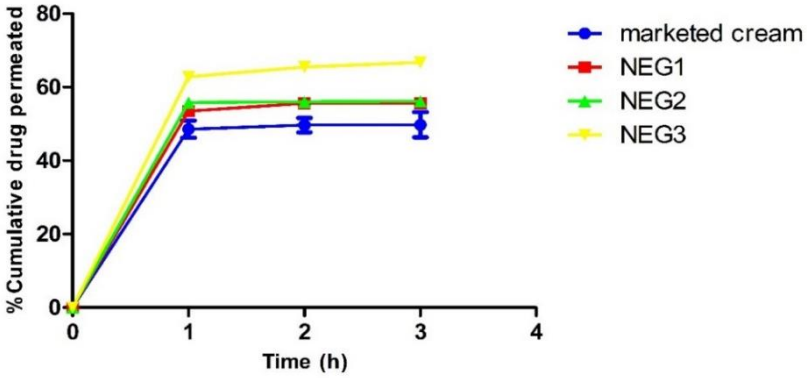

Figure 2. The cumulative percentage of SFP permeated from NEG1, NEG2, NEG3 and marketed cream through snake skin membrane as a function of time.

Table 7. Skin irritation study.

Formula	Time							
	1 h		24 h		48 h		72 h	
	Erythema	Edema	Erythema	Edema	Erythema	Edema	Erythema	Edema
NEG_1	0	0	0	0	0	0	0	0
NEG_2	0	0	0	0	0	0	0	0
NEG_3	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

NEG_1: 0.125% SFP, NEG_2: 0.25% SFP, NEG_3: 0.5% SFP
Positive control: 2% (w/w) SFP, negative control: no application
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

Table 8. The average of burn wound area measurement on rabbits.

Treatment	The average of wound area on the day (cm^2)									
Groups	0	1	2	3	4	5	6	7	8	9
G1	2.5±0.3	2.5±0.2	2.35±0.1	2.2±0.3	2.05±0.1	1.95±0.2	1.85±0.3	1.75±0.1	1.55±0.2	1.25±0.3
G2	2.5±0.2	2.5±0.1	2.4±0.5	2.3±0.1	2.15±0.3	2.0±0.5	1.85±0.2	1.65±0.6	1.45±0.5	0.85±0.4*
G3	2.5±0.1	2.5±0.2	2.25±0.2	2.1±0.3*	1.95±0.1	1.8±0.1*	1.65±0.3*	1.45±0.1*	1.15±0.7*	0.45±0.2*
G4	2.5±0.2	2.5±0.4	2.4±0.5	2.4±0.1	2.3±0.1	2.2±0.2	2.1±0.4	2.0±0.3	1.9±0.1	1.6±0.5
G5	2.5±0.3	2.5±0.5	2.5±0.2	2.4±0.5	2.4±0.1	2.3±0.3	2.3±0.1	2.2±0.6	2.1±0.3	2.0±0.2

G1: The treatment group I (NEG_1: 0.125% SFP)
G2: The treatment group II (NEG_2: 0.25% SFP)
G3: The treatment group III (NEG_3: 0.5% SFP)
G4: The treatment group IV (marketed cream: 2% SFP)
G5: The treatment group V (negative control: NEG basis)
*P<0.05; One Way ANOVA Test, the value of P or Sig. 0.031 which means smaller than α 0.05.

Otherwise, group III showed burn wound area change which, given NEG_3 0.5% gave a change in wound area significantly from 2.1 to 0.45 cm^2 which was faster than other groups. This change was characterized by emerging new granulation tissue on the burn wound edge and the wound had been dry on the 9th day. Furthermore, positive control containing snakehead fish cream 2% (marketed cream) and negative control (nanoemulgel basis) had healing process which was slower than the treatment groups around two weeks, which marked the wound area change from 2.3 to 1.6 cm^2 for marketed cream and 2.4 to 2 cm^2 for basis nanoemulgel.

Discussion

In the formulation of nanoemulsion as a topical drug delivery faces many challenges in delivering drug effectively through the skin, which rates controlling barrier for topical drug delivery.[35] Small particle sized formulation and rheology properties are very important to deliver drugs through the skin. Therefore, this study is done formulation development of snakehead fish powder (SFP) into nanoemulsion using the selection of a suitable oil, surfactant and co-surfactant.

According to Tungadi (2011) stated that snakehead fish powder which designed into cream dosage form showed low stability after several months storage due to droplet size of emulsion (20 µm). [36] Regarding this, SFP was formulated into nanoemulsion based on the solubility studies in different oils, surfactants and co-surfactants. Olive oil was selected as the oil phase, whereas a non-ionic surfactant, Tween 80, was selected as surfactant from SFP nanoemulsions having the least toxicity and irritant potential as compared to ionic surfactants, and having a low critical micelle concentration.[37] Propylene glycol was selected as the co-surfactant having a decisive role during nanoemulsion formulation by decreasing the interfacial stress, leading to interfacial film flexibility and also as penetrant enhancer. It can be observed that at Smix ratio of 4:5, 4.5:5.5, 5:6 and 5.5:6.5 (NE1-NE4: Figure. 1), an area of nanoemulsion in the phase diagram.

Nanoemulsions are kinetically stable systems and are formed by using a particular concentration of various components, with no sign of phase separation, creaming or cracking under various stress conditions. The most important feature of nanoemulsions is the mean droplet size, which must be in the nanometric range.[18] The low pressure influence of magnetic stirrer can reduce droplet size of nanoemulsion to achieve the mean droplet size of less than 200 nm, was studied.

Nanoemulsions developed with non-ionic surfactants were stabilized mainly via steric mechanism which Tween 80 as a non-ionic surfactant has high hydrophilic and lipophilic balance (15) so that it can be stable in an emulsion system with oil in water.[38] This surfactant has pivotal roles in nanoemulsion basis because it has a large surface area for reducing interfacial and surface tension causing the surfactant to be absorbed on interface phase. Regarding this, it can decrease the surface free energy by ruining globule and resulting small globule.[39] The most surfactants are not able to reduce interfacial tension in emulsion so that it needs to add co-surfactant (propylene glycol) which can increase the solubility of nonpolar groups.[40] Besides that, it can intensify flexibility of surfactant film and fluidity of emulsion phase.

Furthermore, the origin of the negative zeta potential of formulations might be due to preferential adsorption or desorption of electrolyte ions on the surface.[41] With increasing the concentration of Tween 80, an increase in the negative value of zeta potential might be associated with the presence of impurities, i.e. peroxides, free fatty acids and so on.[42,43]

Besides that, small particle sized formulation yet concerned when delivering drugs through the skin, the rheology properties of nanoemulsion is imperative. The nanoemulsion formulation, it may be not advantageous to make utilized because of low viscosity and spreadability. Therefore, the approach of incorporation of nanoemulsion with the gelling system can help in overcoming this problem. Meanwhile, the previous research about nanoemulgel of SFP contained hydrophilic compounds such as albumin showing a different formulation data for size, PDI, and zeta potential. Otherwise, this research showed a nanoemulgel data for characterization and optimization of SFP containing hydrophobic compounds such as arachidonic and eicosanoic acids for accelerating burn wound healing process by transdermal delivery.

Among different nanoemulgels, $NEG_{1.5}$ was found to be a creamy and viscous preparation having a smooth homogenous texture, glossy appearance and no sign of phase separation. Whereas $NEG_{2.0}$ and $NEG_{2.5}$ was too viscous and turbid in appearance based on visual appearance and viscosity. HPMC 1.5% as gelling agent has low viscosity so that it is easy for SFP to penetrate into skin cell membrane. Otherwise, the other concentrations had high viscosity which can affect SFP penetration into skin cell membrane. HPMC has the ability to spread better than carbopol, methylcellulose, and sodium alginate and is also easy to apply to skin.[44,45] Besides that, the advantages of HPMC are neutral, stable viscosity, resistant to microbial growth, clear gel and strong film on the dry skin.[46]

The greatest obstacle upon transdermal drug delivery refers to barrier properties of stratum corneum a 10 µm to 20 µm thick tissue layer with great composed structured lipid/protein matrix.[47] Adopting Tungadi (2016), it can be concluded that SFP cream containing penetrant enhancer i.e. propylene glycol could accelerate wound healing process by skin permeation study, but the cumulative of penetrating albumin into rat skin was only 49%.[1] Meanwhile SFP nanoemulgel can improve the permeation of drug through the skin, which can be seen from the cumulative percentage of SFP permeated of NEG3 (0.5% SFP) was the highest (66.75±1.03%) through a snake skin membrane compared to the positive control (marketed cream) only 49.80±3.42%. It means that the formulation of nanoemulgel for the topical delivery system acts as drug reservoirs which, influence the release of drugs from the inner phase to the outer phase and then further onto

the skin.[48] These release mechanisms depend on the composition of the network polymer chains and the Crosslink density.[49] Besides that, the ability of a drug to permeate the skin and successfully release of therapeutic agent is influenced by drug affinity to diffuse out from the vehicle and permeate through barrier.[50]

Skin irritation studies conducted on rabbits showed that during 3 day observation period, no evidence of irritation (erythema and edema) was observed on visual inspection after the application of nanoemulgel on rabbit skin for all formulations of NEG1, NEG2 and NEG3. Thus, the developed formulation was non-sensitizing and safe for topical use.

Based on observations can be seen that on 3rd, 6th, 9th day, burn wound on the rabbit treatment of group III, which given NEG3 0.5% SFP experienced a significant reduction in wound area faster than other groups which marked by the presence of fibrin yarns covering the burn wound. This change was characterized by emerging new granulation tissue on the burn wound edge and the wound had been dry on the 9th day. Furthermore, positive control containing snakehead fish cream 2% (marketed cream) and negative control (nanoemulgel basis) described a healing process which was slower than the treatment groups around two weeks. NEG3 is the best formula of SFP nanoemulgel in vivo study because nanoemulgel is a transdermal delivery system providing a controlled release of controlled substances over a period of time and improve patient comfort during dosage preparation. Small droplet size can absorb albumin having large molecules which will follow the size of the globule spontaneously and surroundings.[51] In addition, gelling agent of nanoemulgel is expected to be able to give a cooling effect on the burn wound.

Besides that, Nanoemulgel on intact with skin will release the oily droplets from the gel network. The oil droplets then will penetrate into the stratum corneum of the skin and directly deliver the drug molecules without a transfer via hydrophilic phase of nanoemulsions.[48] Moreover, the formulated nanoemulgel system was found to possess good permeation potential without the incorporation of any chemical enhancers which are habitually irritants.[52] Hence, the novelty of this system lies here, as the components (oil, surfactant, especially cosurfactant) of nanoemulgel themselves acted as permeation enhancers. Besides that, NEG of snakehead fish containing hydrophobic powder has higher permeation compared to marketed cream of snakehead fish around 1.5 times. Otherwise, the previous research lacks of information about the application of nanoemulgel only characterization, optimization and was not stable.[53]

Based on Anova One Way analysis results stated that the value of P or Sig. 0.031 meaning smaller than α 0.05. It can be concluded that there is a very significant difference between the average of burn wound area on rabbits. This means that SFP can be designed in a nanoemulgel dosage form giving a significant effect on each treatment. Thus the nanoemulgel formulation could be beneficial in improving bioavailability and permeation of SFP for transdermal delivery particularly burn wound healing.

Conclusion

It can be concluded that the novel nanoemulgel of snakehead fish powder with suitable viscosity was successfully formulated for transdermal application based on the results of evaluations and stability tests. They showed snakehead fish nanoemulgel had good stability, including the characterization of snakehead fish nanoemulgel having functioned as topical delivery based on the significant higher permeation profile (1.5 times higher than marketed cream) including the accelerating of burn wound healing in vivo study.

Conflict of interests

The authors claim that there is no conflict of interest.

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