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Evaluation of Diabetic Wound Healing Activity of Novel Quercetin Topical Preparations

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ABSTRACT

Diabetic wound needs effective pharmacotherapy in order to accelerate the wound healing process and to prevent further infection. Quercetin (QUE) is one of flavonols which is potential as an active ingredient for diabetic wound therapy, due to its anti-inflammatory and antioxidant activities. This current study evidenced wound healing activity of QUE topical preparations on the open wound of diabetic-induced subjects preclinically. The investigation focused on topical administration of QUE nanoemulgels and QUE micellar gels (0.2% w/w of QUE) on groups of diabetic-induced male Wistar rats. The quality of preparations in terms of organoleptic performance, pH, viscosity and spread-ability was physically characterized prior to topical administration to the wound. Punch biopsy (5mm) was applied to create the open wound. The visual observation of wound contraction area (in mm^2) was carried out every three days. On the day 12th, after sacrificing the subjects, the wounded tissues were removed, fixed in 10% formalin and prepared for histology examination-hematoxylin and eosin staining. The results showed that the QUE nanoemulgels and QUE micellar gels were relatively comparable on the physical quality as well as the wound healing activity. Their wound healing activities were remarkably faster compared to the untreated control group and it was showed from the histology study that the QUE nanoemulgels and QUE micellar gels successfully accelerate the wound healing process to the proliferation step if it was compared the untreated. In conclusion, QUE nanoemulgels and QUE micellar gels were potential to develop to accelerate the diabetic wound healing process.

INTRODUCTION

The Diabetic open wound, is one of problematic pathologic conditions in diabetes mellitus with severity-increasing risks due to the uncontrolled blood sugar level and poor blood circulation (Burgess *et al*., 2021; Greenhalgh, 2003). Untreated diabetic wound which is mostly located on feet manifests in the chronic inflammation due to the increase of inflammatory cells which also generate excessive reactive oxygen species (ROS) and introduces matrix metalloproteinase, which subsequently increases the oxidative stress and reduce the extracellular matrix as well as growth factors (Chang, 2016; Chen *et al*., 2023; Deng, *et al*., 2021). This condition, complicated by peripheral neuropathy, may exacerbate into tissue death and foot ulcers leading to an amputation and significantly reduced quality of life (Deng, *et al*., 2021; Jalilian *et al*., 2020; McKittrick, *et al*., 1949).

Ghobadi *et al*. (2020) noticed that the foot ulcers therapeutic cost has showed deep impact on the economic values and the healthcare system, as it was predicted to affect 24.5% of the total healthcare cost with the values closed to $ 11 billion (US) and $ 450 million (UK). Therefore, diabetic open wound needs more intensive wound care in order to avoid the worse condition such as gangrene infection, which may lead to

foot ulcer and eventually amputation (Jalilian *et al*., 2020; Tuglo *et al*., 2022).

Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one; MW 302.236; QUE), one of flavonols extracted from many varieties of fruits and plants, has displayed many pharmacological activities (Azem *et al*., 2023; Li *et al*., 2016). It also has been widely studied as the wound healing substances (Ahmed *et al*., 2018; Jangde *et al*., 2018; Lesjak *et al*., 2018; Taskan *et al*., 2019). QUE plays an important role in wound contraction by demonstrating significant anti-inflammatory activity (Beken *et al*., 2019; Hou *et al*., 2019; Lesjak *et al*., 2018) and antioxidant action in alleviating the oxidative stress (Ahmed *et al*., 2018; Beken *et al*., 2019; Deng, *et al*., 2021; Kant *et al*., 2021). QUE orchestrates up-regulating of growth factors including VEGF as well as down-regulating of pro inflammatory cytokines (TNF-α, IL-1β) and proteolytic enzymes including matrix metalloproteinases-MMP 1 and 9 (Chen *et al*., 2023; Yan *et al*., 2022).

To accelerate the recovery process of diabetic wound healing, topical preparations of QUE are the best choice and they need to be formulated in a good quality and efficacy. Although pharmacological benefits of QUE have been well reported (see above), the efficacy of any topical preparations on the open wound of the diabetic subjects is sought to be examined in order to provide adequate medication in accelerating diabetic wound healing.

A nanoemulgel system is a physical synergistic combination of nanoemulsion and a gel system creating a semisolid system (Bashir *et al*., 2021; Ghareeb, 2019; Nastiti at al., 2023; Phaugat *et al*., 2022). Nanoemulsion offers opportunities to increase the solubility of lipophilic agents (Jadhav *et al*., 2020; Nastiti *et al*., 2017; Tayeb *et al*., 2021). The stability of the natural compound along with acidic pH conditioning in the product was also successfully maintained by nanoemulsion formulation (Nastiti *et al*., 2020). Nevertheless, low viscosity of nanoemulsions occasionally limits the ease of nanoemulsion topical application (Nastiti at al., 2023). The incorporation of gelling agent results in better performance of nanoemulgel in terms of better spread-ability and physical stability of the product. Micellar gels are the formulation of micelle-rich hydrogel. Self-assembly micelles due to exceed concentration of surfactant (above the critical micelle concentration; CMC) works well in solubilizing the lipophilic compound (Sharma, *et al*., 2023) in the system to provide homogenous mixture in the hydrogel. Polymeric micelles have also well contributed to the wound healing formulation (Barroso *et al*., 2020). The use of non-ionic surfactant in the micelle formation features minimal risk of irritation and pain sensation (Alberti *et al*., 2017).

On this study we focused on the diabetic wound healing activity of two types of topical preparation which were nanoemulgels and micellar gels. With the incorporation of QUE, we aimed to examine the potential healing process of those preparations on the open wound of diabetic-induced subjects preclinically.

METHODS

Material

QUE with ≥ 95% purity, triacetin, Kolliphor ® RH 40, Transcutol® and other chemicals were purchased from Sigma-Aldrich (Singapore), carboxymethylcellulose Na was obtained from Dwi Lab Mandiri_Scientific (Indonesia), Lukajel® (Indonesia).

QUE nanoemulgel and QUE micellar gels fabrication

In the nanoemulgel fabrication, triacetin, Kolliphor® RH 40 and Transcutol® were mixed with water to create transparent nanoemulsion. QUE was then dissolved, followed by the addition of CMC Na. The mixture was stirred at 200 rpm for 5 minutes and kept out of light over 24 hours to allow the nanoemulgel formation.

Table 1. Formulation of topical preparation (in 100 grams)

Ingredients	QUE nanoemulgels	QUE micellar gels
Quercetin	0.2	0.2
Triacetin	10	-
Kolliphor® RH 40	21.5	21.5
Transcutol®	14	14
BHT	0.6	0.6
Nipagin	0.2	0.2
Na CMC	3	3
Aquadest	50.5	60.5

In terms of micellar gel formulation, Kolliphor® RH 40 and Transcutol® were mixed at 100 rpm for 5 minutes and this mixture was used to solubilize QUE. The hydrogel was prepared by swelling the CMC Na in water for 24 hours. The mixture of surfactant-quercetin then was added in the hydrogel and stirred at 50 rpm for 5 minutes to achieve homogenous system of micellar gel.

QUE micellar gels and QUE nanoemulgel were prepared by incorporating 0.2% quercetin (Table 1).

Quercetin topical preparation characterization

The characterization of QUE micellar gel and QUE nanoemulgel involved visual organoleptic observation (color, texture, and odor), pH confirmation, viscosity determination and spread-ability examination. Physical stability of the topical preparations was also assessed in terms of viscosity alteration on real time storage (14-day), protected from light and at room temperature.

The pH of the topical preparations was confirmed by measuring the pH of preparations using pHmeter (Ohaus, USA) at room temperature. The pH must be in the range of 6-6.5.

Determination of viscosity of the QUE topical preparations was carried out at room temperature using a Merlin VR viscometer (Rheosys, USA) (Nastiti *et al*., 2023). The operational mode setting was cone and plate 2°/30 mm with the rate of 50 rpm. Four replicates of each formula were measured with a delay time of 20 seconds, zero-shear time of 20 seconds and the integration time of 10 seconds. The viscosity acceptance value was at 1.5-2.5 Pa.s.

Spread-ability was examined by measuring spreading diameter of one gram of topical preparation, after being placed in between two glass plates for 1 min, with the upper plate weighed 125g (Garg *et al*, 2022). Four plots of diameter measurements were taken to achieve the average of spreading diameter. The expected diameter for good spread-ability was at 5-7 cm.

Physical stability of topical preparations was observed by comparing viscosity of the preparations 24 hours after fabrication to the one on day 14th which stored at room temperature. The viscosity should not alter above 20% during the storage at room temperature.

Overall acceptance profiles of the topical preparations were yellowish semisolid appearance with pH of 6-6.5, viscosity of 1.5-2.5 Pas, spread-ability of 5-7 cm and ≤ 20% alteration of viscosity (physical stability).

Experimental design of the study of wound healing activity of the topical preparation

The ethical clearance was approved by the Ethic Committee of Faculty of Medicine, Public Health and Nursing, Gadjah Mada University, Indonesia on June 7th 2023, number KE/FK/0946/EC/2023. Healthy male Wistar rats aged 2.5-3 month (200g-250g BW) were housed individually and acclimatized under 12h light/dark cycles at room temperature and 70% RH. The rats were fed with standard nutritious pellets and were allowed to drink water ad libitum.

Diabetic induction was carried out on the rats by administering Streptozotozin (45mg/kg BW) and Nicotinamide (110mg/kg BW) intraperitoneally. After 72 hours, the blood glucose level was measured using Glucose-Oxidase Peroxidase (GOD-PAP) method to ensure that the rats had been diabetic (blood glucose level at ≥ 250 mg/dL). The blood sugar level of the subjects was also confirmed on the day 12th of visual observation.

The diabetic-induced rats then were divided into 4 (four) groups @3 rats. The first group (G1) was the untreated control group. The positive control group was treated with the commercial product (Lukajel®) (G2). The other groups were the group treated with QUE nanoemulgel (0.2%) (G3), and QUE micellar gel (0.2%) (G4).

All rats were anaesthetized by intramuscular injection of ketamin hydrochloride (50mg/kg BW) and xylazin (10mg/kg BW). The hairs on the dorsal surface were removed prior to wound formation. Punch biopsy with diameter of 5mm and the depth of approximately 2 mm was carried out twice on each side of the back of rats after being sanitized with ethanol 70%. Approximately 200 mg of the topical preparation was then applied on the wound with the aid of a metal mini-spatula, twice a day for 11 consecutive days (Veronica and Dwiastuti, 2022).

The observation of the wound contraction was carried out every three days: 3rd, 6th, 9th and 12th day. The wounds were captured perpendicularly using a digital camera (Samsung Galaxy S23, South Korea) at 20 cm height, with the scaling aid of millimeter-scale paper (1 scale

bar = 5 mm). The area of the wounds then was analyzed using ImageJ software (National Health Institute, U.S). The percentage of wound contraction was calculated by: [(wound area on the day 0 – wound area on the day X): wound area on the day 0] (Kant *et al*., 2021).

On the 12th day, after being visually observed, all rats were sacrificed using excessive dose of ketamine injection peritoneally (200mg/kgBW). The wounded skin tissues were then removed and fixed in 10% formalin for histological observation.

Fixed tissues were further embedded in paraffin, sectioned at the thickness of 5µm using a microtome, then stained with hematoxylin and eosin. The sections were examined under a light microscope for the evaluation of histopathological parameters. Two parameters were selected, those are the number of inflammatory cells (represented by neutrophils) and the number of vessels generated (angiogenesis). The scoring was carried out based on the score system developed by Hazrati *et al*. (2010).

Data presentation and statistics analysis

The data are presented as the mean ± SD for physical characteristics with n=4 replications/formula and the mean ± SEM for wound healing activity study with n=6 wounds/group. Normally distributed data was analyzed using either ANOVA or unpaired t test. For the non-parametric data, a Wilcoxon test and Kruskal Wallis were applied. Significant differences were considered if $p < 0.05$. All data were statistically analyzed using Analysis-ToolPak on the MS Excel 2019- (Microsoft, USA). In terms of the wound healing activity study, comparisons were made between topical preparations and control groups and between micellar gel and nanoemulgel groups.

RESULTS AND DISCUSSION

In order to commercialize topical wound healing products, semisolid preparation must provide safety, good spread-ability and bio-adhesivity, adequate capability to deliver the medication, convenience and relatively unproblematic-scaling-up fabrication (Ilić *et al*., 2021; Namjoshi *et al*., 2020). Ultimately, the efficacy of the products needs to be confirmed. Therefore, in this current study, low-energy processing topical formulations incorporating QUE as the active ingredient were challenged to undergo in vivo investigation on the open-wound healing activity on diabetic-induced rats. The difference of those two formulations was the existence of triacetin as the oil phase in the QUE nanoemulgel formulation. Triacetin, a triglyceride plasticizer was also reported to have antibacterial effect (Darabian *et al*., 2020; Fiume, 2003), therefore it was expected to support the wound repairing process. Sodium carboxymethyl cellulose was applied as the gelling agent as it withstands a wide range of pH condition and is highly absorptive (Darabian *et al*., 2020).

The nanoemulsification yielded quite viscous, transparent liquid systems indicating the nano-size performance of droplets and QUE complete solubilization in the system (Figure 1).

Ultimately, to improve the spread-ability of nanoemulsion, the formulation was formed to semisolid preparation with the aid of CMC Na as the three-dimension-structure support system.

The formulation of QUE nanoemulgel and QUE micellar gel provides good characteristics of topical preparation and met the acceptance criteria. Both QUE nanoemulgels and QUE micellar gels were showing smooth, homogeneous yellowish semisolid preparations with pleasant odor (Figure 2).

Figure 1. QUE nanoemulsion

Figure 2. QUE nanoemulgel (a) and QUE micellar gel (b)

Table 2. Characterization of topical preparation ($\bar{x}$ ± SD, 4 replicates)

Physical characteristics	QUE nanoemulgels	QUE micellar gels
pH	6.12 ± 0.05	6.6 ± 0
Viscosity (Pa.s)	2.01 ± 0.08*	1.90 ± 0.15*
Spread-ability (cm)	3.93 ± 0.88*	4.67 ± 0.05*
Viscosity alteration (%)	9.19**	15.45**

Note: * p>0.05
** calculated from the average value

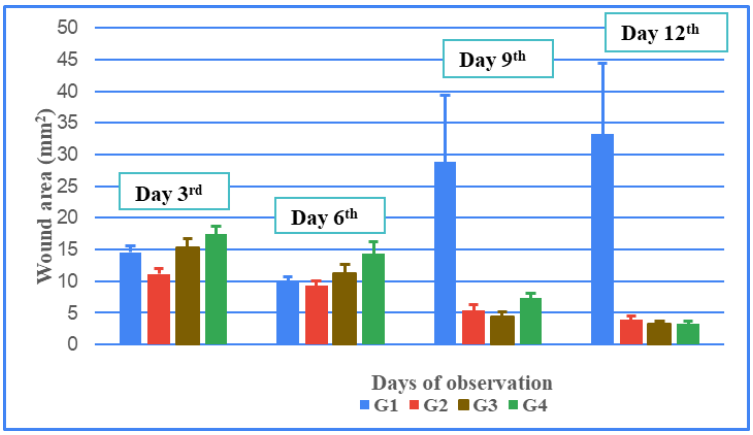

Figure 3. Area of the wound of diabetic-induced rats in 12 days of visual observation (mm²; $\bar{x}$ ± SEM, 6 wounds/group): G1-untreated control group; G2-positive control group; G3-QUE nanoemulgel and G4-QUE micellar gel.

Table 3. Blood glucose level of diabetic-induced rats during observation ($\bar{x}$ ± SD, 3 subjects/group)

	Blood glucose level (mg/dL)			
	G1	G2	G3	G4
Initial check	269.07±7.66	260.05±5.32	264.38±2.11	270.04±3.82
Final check	271.85±8.01	262.90±4.75	266.43±2.45	272.08±3.24

Note: diabetic condition was confirmed at blood glucose level ≥ 250 mg/dL
G1: untreated control group; G2: positive control group; G3: QUE nanoemulgel; G4: QUE micellar gel

Physical characteristics of QUE nanoemulgels was as good as QUE micellar gels in terms of pH and the spread-ability (Table 2). However, the viscosity of the nanoemulgels was higher than the micellar gels, resulted in the more rigid structure, hence the more physically stable semisolid system. The presence of triacetin as the oil phase and particularly its interaction with other components in the nanoemulgels was predicted to increase the viscosity as a result of emulsification due to the significant droplet size reduction (Bahloul *et al.*, 2023).

The in vivo wound healing activity was examined on the open wound of diabetic-induced male Wistar rats. The examination was carried out by visually measuring the area of the wound periodically, and establishing histology assessment on the wound condition on the day 12th of observation.

Figure 4 depicts the progress of wound area in the 4 (four) groups (G1-G4) of the in vivo study of wound healing process from day 3rd to 12th. All treated groups (G2-G4) showed remarkable reduction of wound area on the day 12th, whereas the untreated group exhibited significant enlarging area of the wound. This indicates that on the untreated skin, particularly in subjects with high glucose blood level (Table 3), the inflammation becomes excessive and it may lead to chronic and severe infection (Figure 4a).

On the other hand, the topical treatment of QUE nanoemulgels and QUE micellar gels decreased the inflammation, resulted in the significantly reduced area of the wound by around 80% on the 12th day (Table 4; Figure 4). QUE nanoemulgels and QUE micellar gels showed similar results of reduced wound area. It was noticed previously that both types of preparation were also showing similar physical characteristics (Table 2),

Histology study of the H&E stained of wounded skin sections was carried out based on 2 (two) parameters which were indicating inflammation stage (number of neutrophils) and proliferation stage (number of blood vessels; angiogenesis). Number of neutrophils and blood

vessels were simplified to a scoring system. According to the Hazrati's scoring system (Hazrati *et al*., 2010), the larger number of inflammatory cells, the smallest score would be, while for the angiogenesis, the number of vessels and the score are in linear correlation. Score 0 indicates more than 15 neutrophils but no vessels detected.

From the Table 5 we can identify that on the untreated group (G1), there were still abundant inflammatory cells (neutrophils) on the day 12th (score 0). Nevertheless, the QUE nanoemulgel group (G3) and the positive control group (G2) showed comparable results (score 3) whereas the least number of inflammatory cells was in the group of QUE micellar gel treatment (score 4). In terms of angiogenesis, number of blood vessels was high on the treatment of QUE topical preparations (G3-G4), even above the number of vessels on the positive control group (G2).

Wound healing normally proceeds in 4 (four) stages: hemostasis, inflammation, proliferation and re-modelling of the skin (Falanga, 2005; Sorg *et al*., 2017; Xu *et al*., 2020). Hemostasis stage occurs in the beginning of the open wound. It is typically signed by bleeding which followed by blood clotting and thrombus formation. The next stage is inflammation, which naturally occurs as a self-defense response on trauma, showing by the presence of many various inflammatory cells including neutrophils. The third stage called proliferation, which is a stage of tissue reconstruction, in which angiogenesis and epidermal resurfacing take place. The last part of wound recovery is re-modelling of collagen into a more organized structure along with the apoptosis process of the unused cells. This stage may take 3 weeks until a couple of months to get fully recovered.

Table 4. Wound contraction on the diabetic-induced rats ($\bar{x}$ ± SEM, 6 wounds/group)

	Wound contraction (%)			
	G1	G2	G3	G4
Day 3rd	25.60±5.16	43.11±4.66*	21.47±7.11*	11.26±6.39*
Day 6th	48.70±3.08	52.62±3.74	42.60±7.36	26.82±9.82*
Day 9th	(-)47.02±53.32	72.49±4.78*	77.39±3.36*	62.73±4.07*
Day 12th	(-)53.00±43.55	79.96±3.12*	83.02±1.84*	83.31±1.88*

Note: * p<0.05, compared to untreated group (G1)
(-) wound enlargement
G1: untreated control group; G2: positive control group; G3: QUE nanoemulgel; G4: QUE micellar gel

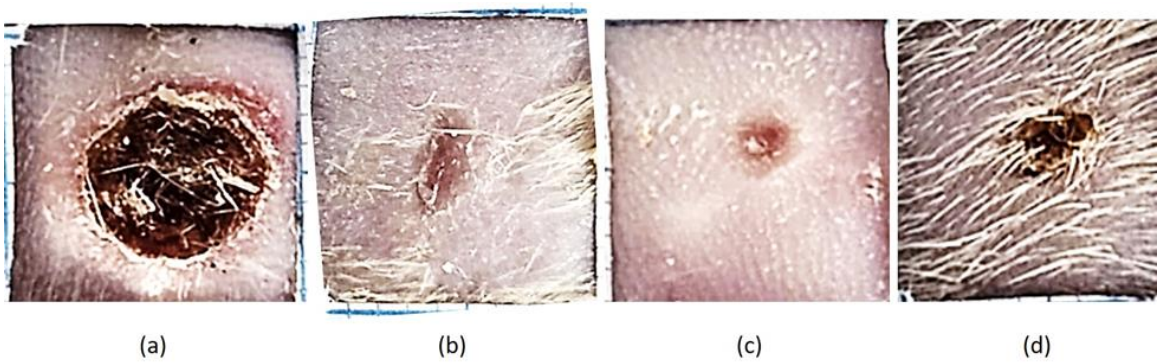

Figure 4. Representative images of visual examination of the wound on the day 12th: G1-untreated control group (a); G2-positive control group (b); G3-QUE nanoemulgel (c) and G4-QUE micellar gel (d). Height of image capturing: 20 cm. Scale bar: 5mm.

Table 5. Histology parameter scores of the groups

Group	Inflammatory cells	Angiogenesis
G1	0	0
G2	3	3
G3	3	4
G4	4	4

Notes: The score list was adapted from Hazrati *et al* [32]. The higher the score of inflammatory cells determined the smaller number of the inflammatory cells, whereas the higher score of angiogenesis reveals the larger number of vessel proliferation.

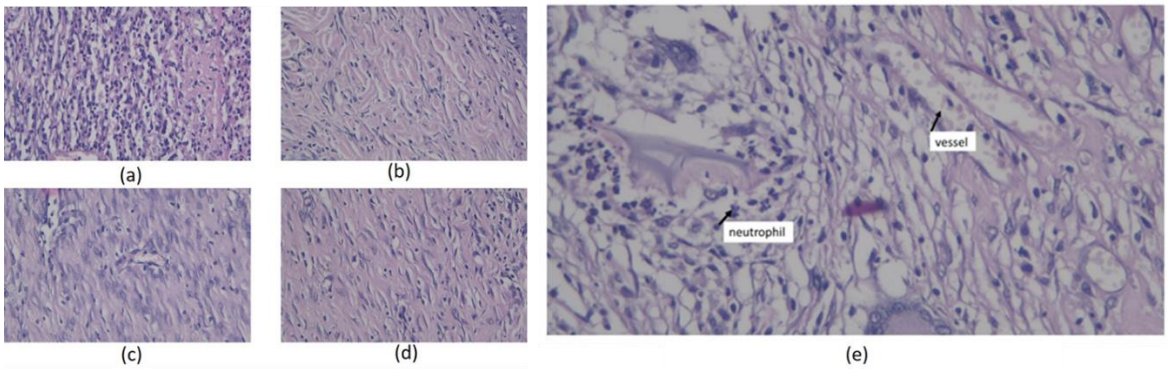

Figure 5. Representative images of H&E staining of dermal site of wounded skin section on the day 12th observation (100x magnification): G1-untreated control group (a); G2-positive control group (b); G3-QUE nanoemulgel (c); G4-QUE micellar gel (d); neutrophil and vessel forms (e)

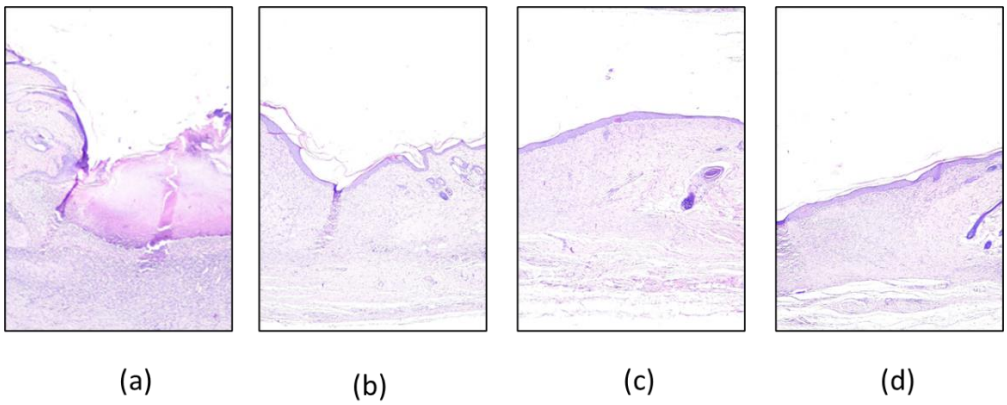

Figure 6. Representative images of cross-sectional wounded skin on the day 12th observation (H&E; 40x magnification): G1-untreated control group (a); G2-positive control group (b); G3-QUE nanoemulgel (c) and G4-QUE micellar gel (d)

Figure 5 shows that on the day 12th the untreated group was still in the inflammation stage, indicated from the existence of numerous neutrophils in the tissue, whereas all treatment groups (G2-G4) appeared to be in the proliferation stage evidenced by the formation of pink and uneven granulation tissues and the blood vessel generation.

In terms of epidermis regeneration (Figure 6), steady growth of epidermis (re-epithelization) on skin treated with QUE nanoemulgels (G3) and QUE micellar gels (G4) was well-depicted and it was comparable to the positive group (G2). This emphasized that the treatment groups have already achieved proliferation stage on the day 12th.

The topical application of QUE nanoemulgels as well as QUE micellar gels evidenced fast relief of the open wound on diabetic-induced rats. The results of the current study are in line with the investigation of Kant *et al*[18], on their study of QUE ointment application on the wound of diabetic rats. They highlighted the activity of QUE in increasing wound contraction and growth factor as well as accelerating re-epithelization, angiogenesis. Promotion of myofibroblast formation and neuronal regeneration were also suggested. It was also well presented that the rate of wound recovery of QUE treated diabetic rats was similar to the wound recovery of healthy rats.

Nanoemulgels and micellar gels offer greater biocompatibility and feature high loading capacity, great wound repair accelerator, convenience in use, controlled release and adequate hydration maintenance (Barroso *et al*., 2020). Moreover, topical delivery systems of QUE loaded nanoemulgels and/or micellar gels provide dual modals between QUE and the vehicles which work synergistically in repairing diabetic wound. Therefore, those formulations are potential to develop and establish as part of diabetic wound care system.

CONCLUSIONS

Based on the results of this current *in vivo* study, we conclude that QUE nanoemulgels and QUE micellar gels were successfully providing

qualified formulations with wound contraction at approximately 80% on diabetic-induced rats compared to the initial wound area. The fast and significant recovery leads the potential of both QUE loaded preparations to develop as the wound healing formulation for diabetic open wound, due to the dual mode of QUE pharmacological effects and the fashion of those topical preparations in dressing the wound.

CONFLICT OF INTEREST

All the authors declared there is no conflict of interest.

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