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RESEARCH PAPER

The effect of topical quercetin loaded liposome on pressure ulcer healing in rats

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ABSTRACT

Objective(s): Quercetin antioxidant properties could play an important role in various fields of health. However, its use has been limited because of several disadvantages such as very low solubility in water and high instability in the presence of air, light and heat. Encapsulation of quercetin in nanostructure systems such as liposome may lead to decrease the adverse effects and protect this molecule against degradation. The aim of this study was preparation and *in-vitro* and *in-vivo* evaluation of liposomes for topical delivery of quercetin to improve the pressure ulcers.

Materials and Methods: Liposomal formulations were prepared by fusion method and characterized. The amount of drug retained in and penetrated through mouse skin after 8 hours were determined. Also microscopic and macroscopic examination of laboratory animals was performed.

Results: Encapsulation efficacy of liposomes was in range 64.66-77.83%. Formulation F4 showed maximum drug release in 8 hours and the remaining drug in the skin layers was more than 46%. Histological investigation suggested that F4 and phenytoin 1% cream have the healing effect on the pressure ulcer during 28 day-treatment.

Conclusion: Quercetin liposomes due to its natural structure and minimal systemic absorption and side effects can be a suitable candidate for the treatment of pressure ulcers.

Keywords: *Fusion; Liposome; Pressure ulcer; Quercetin; Topical delivery*

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INTRODUCTION

Flavonoids have a broad pharmacological profile such as anti-lipoperoxidant [1] and anti-inflammatory [2] properties. Quercetin, 3,3′,4′,5′-7- pentahydroxy flavone, a polyphenolic flavonoid, extremely hydrophobic in nature, is a component of onion [3] and is able to interact and permeate lipid bilayer and such capacity is very important, because there is correlation between antioxidant activity and ability to incorporate into membrane [4, 5].

Different studies claimed various mechanisms for wound healing effect of quercetin, i.e. upregulation of vascular endothelial growth factor (VEGF), and interleukin-10 as well as downregulation of tumor necrosis factor-alpha. Increased activity of superoxide dismutase and catalase were also reported [6]. Meanwhile, suppressing mitogen-activated protein kinase (MAPK) is the main mechanism that mostly claimed by numerous studies for several properties of quercetin [7-9].

The skin is the largest organ of the body, accounting for about 15% of the adult body's weight. Protecting the body against physical, chemical, biological, and body temperature defects is one of the tasks of this organ. The skin consists of three layers that come from the outside: epidermis, dermis and hypoderm [10]. Pressure ulcer is an important cause of morbidity, mortality

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and extended hospital stay among patients with spinal cord injuries [11] or ischemia-reperfusion injury [12]. Ulcer can range from stage 1 with intact skin to stage 4 with full-thickness tissue loss and exposed bone, tendon, or muscle [13]. Pressure ulcer treatment involves various approaches, including interventions to treat the condition that lead to pressure ulcers (support surface and nutritional support), interventions to protect and promote healing of the ulcer (wound dressings; topical applications; and various adjunctive therapies, such as electrical stimulations; light therapy, and vacuum-assisted devices), and surgical repair of the ulcer [14, 15]. Wound healing processes include: 1. Rapid response 2. Inflammatory response 3. Proliferation, migration and contraction-recovery [16].

Liposomes are colloidal carriers containing phospholipids as the main composition of their bilayer structure, which resembles the lipid cell membrane of the human body [17]. Additionally they can load hydrophilic as well as lipophilic drug in their core, and exhibit biocompatibility with low toxicity[18]. Liposomes offer a number of advantages in dermal and transdermal drug delivery as they have a high solubilization capacity and a penetration enhancer effect [19]. So, they are suitable candidate for preparing topical formulations.

In this study, the quercetin liposomal (QL) formulations prepared by fusion method and the effects of these formulations on pressure wound healing in male Wistar rats were evaluated.

MATERIALS AND METHODS

Materials

Soy phosphatidylcholine (phospholin® 85G) was obtained from lipoid (Germany). Quercetin, cholesterol and HEPES (4-(2-Hydroxyethyl) 1-piperazine ethanesulfonic acid) were purchased from Sigma (Germany). Propyl paraben, methyl paraben, teriton X100, propylene glycol and vitamin E were obtained from Merck (Germany). Phenytoin 1% cream was purchased from pharmacy (Daru pakhsh, Iran).

Animals

42 Male wistar rats, 150-200 g weights, obtained from Animal Care and Breeding Center of Ahvaz Jundishapur University of Medical Sciences (Ahvaz, Iran). The experiments were conducted in full compliance with local regulatory principles of ethics committee of Ahvaz Jundishapur University of Medical Sciences (Ethics code: IR.AJUMS.REC.1396.686).

Preparation of liposomes

Liposomes were prepared by fusion method in different concentration of quercetin as stated in our previous study. Briefly, the lipid components consisted of Soy phosphatidylcholine (SPC) (20% w/w), cholesterol (2% w/w), propylene glycol (6% w/w), vitamin E (0.3% w/w), methyl paraben (0.1% w/w), propyl paraben (0.02% w/w) were melted at about 75°C. HEPES buffer (10Mm, pH=5) containing propylene glycol and quercetin in final concentration of 0.001(F2), 0.002(F3) and 0.004(F4) g/ml was heated separately and was added up to 100% to the previously heated melted lipids, and the mixture homogenized with a homogenizer (Ultra-Turax IKA T25) for 5 min at 12000 rpm and allow it to cool down to room temperature[20].

Characterization of liposomes

Particle size

The particle size of the samples was measured in triplicate by laser scattering (Scatterscope 1, Qudix, South Korea). Samples diluted in HEPES buffer to a suitable concentration (0.2g formulation in 1ml HEPES buffer).

Encapsulation efficacy

Encapsulation efficacy of liposomes was determined directly. Certain amounts of liposomal dispersion were centrifuged (VS-35SMTI, Korea) at 20,000 rpm for 25 min at 25°C. Precipitated pellets were re-dispersed in 2 ml triton 10 X and the final clear solution was analyzed for quercetin content using a UV spectrophotometer at 369 nm[21]. Samples were diluted if needed to get absorbance in range of prepared standard curve.

$$\%EE = \frac{amount\ of\ drug\ in\ precipated\ pellets}{total\ drug} \times 100$$

In vitro drug release

Drug release studies were performed using dialysis membrane method. Dialysis membranes were soaked in distilled water for 20h before use. 1g of formulation was placed in a dialysis membrane and both ends were closed. The membrane was float in a beaker containing 150ml phosphate buffer (pH 7.4) and ethanol (3:1 v/v)

and stirred at 200 rpm in 37°C. 1 ml of receiver medium was removed at 15, 30, 45, 60, 90, 120, 180, 240, 360 and 480 min and same volume of fresh medium was replaced. The samples were analyzed for their quercetin content. The receiver concentration values were corrected by using the equation 1: [22]

$$Mt(n) = Vr^{*} Cn + Vs^{*} \Sigma Cm \quad (1)$$

Mt (n) is the current cumulative mass of drug transported across the membrane at time, n is the number of sampling, Cn is the current concentration in the receiver medium, ΣCm is the summed total of the previously measured concentration, Vr is the volume of the receiver medium, and Vs correspond to the sample removed for analyzed [23, 24].

Stability

The formulation were stored at refrigerate (4 °C) for 3 months and the particle size and encapsulation efficacy of the formulations were measured and compared with those at the time of preparation [25].

Viscosity

Viscosity of formulations were measured by Brookfield viscometer (DVII + Pro, US) at 10 rpm and 25°C using spindle of 64.

Differential scanning calorimetry (DSC)

DSC thermograms of SPC, cholesterol and formulations were recorded on a differential scanning calorimeter (Mettler Toledo, DSC-1, Switzerland). Thermograms of both blank and QL were recorded individually. Certain amount of sample was placed in aluminum pan and scanned from 20 to 200 °C by scanning rate of 10 °C/min.

In vitro skin penetration and retention

In vitro skin penetration studies were performed using jacketed Franz cells with a receiver medium of 25 ml Phosphate buffer (pH 7.4) and ethanol (3:1 v/v) at 37 °C and surface area of 5 cm2. The dorsal skin of mouse was shaved with electric clippers one day before the experiment. A suitable size of full-thickness skin of mouse was cut and clamped between the donor and receiver compartment of Franz cell with the stratum corneum side facing upward. The skin samples were initially left in the Franz cells for 30 min in order to facilitate hydration. Subsequently, 1 g of the selected formulations was placed onto the skin surface. 1 ml of receiver solution was removed at 15, 30, 45, 60, 90, 120, 240, 360 and 480 h and same volume of the fresh medium was replaced. The collected samples were analyzed for their quercetin content. The derived concentration values were corrected according to equation (1). For the determination of the amount of the drug retained in the skin, at the end of the experiment, the amount of the formulation remaining on the surface of the skin was collected and assayed for quercetin. The amount of quercetin retained in the skin was then calculated by subtracting the sum of the amount of quercetin that remained on the surface and the amount of quercetin that was released (penetrated through the skin) from the whole applied amount [26].

Animal study

Pressure ulcer model in rats

Animal studies were performed in full compliance with local regulatory principles of ethics committee of Ahvaz Jundishapur University of Medical Sciences (AJUMS) (approval ID: IR.AJUMS.REC.1396.686). Animals were kept in separate cages for two weeks in normal conditions. Access to water and food was unrestricted for them, and 12-hour cycles of light/dark were provided. Pressure ulcer model was developed using a murine model of ischemia and reperfusion-induced skin injury. In brief, after shaving the hair, the back skin of the rats was placed between the clamps for 2 hours followed by 30-min release which was repeated for 4 cycles for 6 consecutive days[21]. At day 7, clear scars were created at the back of the animal.

Administration of QL and macroscopic analysis

Animals are categorized into 6 groups of 7 as below and formulations were used twice daily for 28 days.

1) Group treated with quercetin-free liposomal base
2) Group treated with QL at concentration of 0.001 g/ml
3) Group treated with QL at concentration of 0.002 g/ml
4) Group treated with QL at concentration of 0.004 g/ml
5) Group treated with phenytoin 1% cream
6) Group that does not receive any treatment

The animal was placed in standard mode and photographed from the high level of the wound during different days (0, 3, 5, 7, 9, 11, 13, 15, 17, 19, 23 and 28) in identical conditions, and scar area and wound length were calculated by the Quick PHOTO MICRO 2.3 software and Axio vision rel 4.8 software, respectively for the evaluation of the rate of progression and wound healing [27].

At the end of the experiment, the rats were anesthetized by inhalation of chloroform in a desiccator and sacrificed. 18 mm × 25 mm skin samples were cut from each animal and its tensile strength measured using a Texture Analyser (STM-20, ENG. DESIGN CO. LTD). Each sample was stretched at rates of 20 mm / min, and the maximum tensile and displacement force curves were plotted [28].

Histopathological assessment

For microscopic examination of the wound healing process, samples from the wound site were isolated from each group on day 28 and fixed in 10% formalin. Paraffin-embedded sections (5 micron) were stained with hematoxylin and eosin. The process of tissue re-epithelialization, angiogenesis, edema and fibroblast proliferation were evaluated.

Samples were also examined for organization of collagen fibers using Masson trichrome staining [29].

Statistical analysis

All experiments were done in triplicate and expressed as mean ± standard deviation. One way analysis of variance (ANOVA) followed by multiple comparisons Tukey test and where applicable T-test was used to substantiate differences between groups. Results with $P<0.05$ were considered to be significant. To evaluate the intra-group outcomes over time, repeated measures of variance analysis were conducted.

RESULTS

The aim of the present work was to investigate the ability of a new nanovesicle, to lead quercetin and improve its local bioavailability and comparing the rate of wound healing in the groups treated with QL formulation and phenytoin 1% cream. In this study the fusion method was used to prepare the topical liposomal formulations. This method is free of organic solvent and yield homogeneous liposomes with high encapsulation efficacies. All experiments were repeated three times.

Characterization of the liposomes

Particle size

The mean particle size of the liposomes was in range of 10 to 16 nm as shown in Table 1. ANOVA analysis showed no significant statistical differences between all of formulations ($P>0.05$).

Encapsulation efficacy

The encapsulation efficacy of formulation was in range of 64.66 to 77.83 (Table 1). ANOVA analysis showed no significant statistical differences among all of the formulations ($P>0.05$). The lipophilic nature of quercetin may lead to encapsulation of this drug in between the lipid bilayers. This can explain the high encapsulation efficacy of the quercetin in liposomal formulations [30].

In vitro drug release

The release profile of quercetin from various liposomal formulations was shown in Fig 1. ANOVA analysis at the end of 8th hour showed that the difference is significant among all of formulations ($P<0.05$). At the end of the experiment, F4 released %35.36 ± 0.14 of incorporated quercetin, had maximum release and F3 released %22.96 ± 0.58 of incorporated quercetin which is the lowest release. Analysis of variance (ANOVA) results in a significant difference in percent of release ($P<0.05$) of F2 formulation compared to F3 and

Tabale 1. Particle size (nm) and encapsulation efficiency (%) of liposomal formulations (mean ± SD)

Formulation (quercetin, g/ml)	Average size (nm)	Encapsulation (%)
F1 (non drug)	13.09 ± 4.04	-
F2 (0.001)	10.30 ± 2.41	74.71 ± 13.04
F3 (0.002)	15.46 ± 3.09	77.83 ± 3.24
F4 (0.004)	14.1 ± 0.78	64.66 ± 18.46

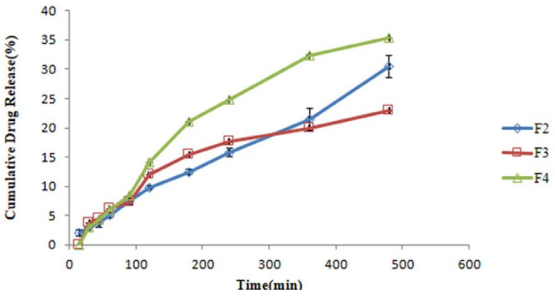


Fig 1. Release profile of quercetin from various liposomal formulations (mean ± SD, n=3)

F4 formulations at 15 min after the start of the experiment, and the difference between F3 and F4 formulation was not meaningful (P>0.05). At 30, 45, 60 and 90 , the percent of release among the three formulations did not differ significantly (P>0.05). At 120, 180 and 240 min, the percent of release was significantly different among all three formulations (P<0.05). There was no significant difference between formulation F2 and F3 at 360 min (P>0.05). At the same time, there was a significant difference in the percent of release of F4 formulation compared to two other formulation (P<0.05). At 480 min, the percent of release was significantly different among all three formulations (P<0.05).

But by assay of quercetin at the end of the test, it was assumed that %58.15 ± 1.65 of quercetin in F2 and %58.53 ± 0.94 in F3 and %51.36 ± 0.68 of quercetin in F4 have been remained in dialysis bag. So it can be concluded that about 15% of released quercetin has been adsorbed by surface of container and/or dialysis bag.

Stability

The results of stability study for formulations are shown in Table 2. T-test results show that particle size of F2 and F3 did not change significantly over the course of three months (P>0.05). This matter could be resulted by the presence of cholesterol that has stabilizing effect against aggregation and fusion of liposomes [31]. The particle size of F4 changes significantly over the course of three months (P< 0.05). T-test results show that encapsulation efficacy of formulations F3 and F4 did not significantly change during 3 month storage (P>0.05). Encapsulation efficacy of formulation F2 was significantly differed during 3 months (P<0.05). That may be due to the lipophilic nature of quercetin that intended to be remained in lipid vesicles.

Viscosity

The viscosity of formulations F2, F3, and F4 were 28491.67±2.08, 41384.00±2.00, and 48048.00±5.29 centipoises respectively. As the results show, the higher the concentration of quercetin in the formulations, the more viscous formulation has been prepared. This result is also consistent with the apparent characteristics of formulations. Analysis of variance (ANOVA) showed a significant difference among all three formulations (P<0.05).

DSC

According to Fig 2. DSC thermogram of cholesterol showed endothermic peak at 140 °C corresponding to its melting point [32]. SPC thermogram showed a broad peak at 88 °C. Quercetin thermogram showed abroad peak at 180-190 °C. In case of QL, the melting point of quercetin was not observed which indicates that it is encapsulated in the liposome [33].

In vitro skin penetration and retention

The results of penetration and retention are shown in Table 3. The results showed that the amount of the drug passed through the skin layers and entered the receptive solution after 24 hours was 6.872 ± 0.009% for F2 and 5.877 ± 0.005% for F3 and 5.192 ± 0.008% for F4. This is an indication that most of the drug has been trapped in the skin layers and not crossed. The goal of this study was to prepare a formulation to provide the highest drug accumulation in the skin layers and restricted

Table 2. Particle size (nm) and encapsulation efficiency (%) of liposomal formulations after 3 months storage (mean ± SD, n=3)

Formulation(quercetin, g/ml)	Average size (nm)	Encapsulation (%)
F2(0.001)	8.13 ± 0.30	81.89 ± 10.46
F3(0.002)	24.56 ± 10.08	82.84 ± 16.51
F4(0.004)	39.83 ± 5.36	80.2 ± 15.14

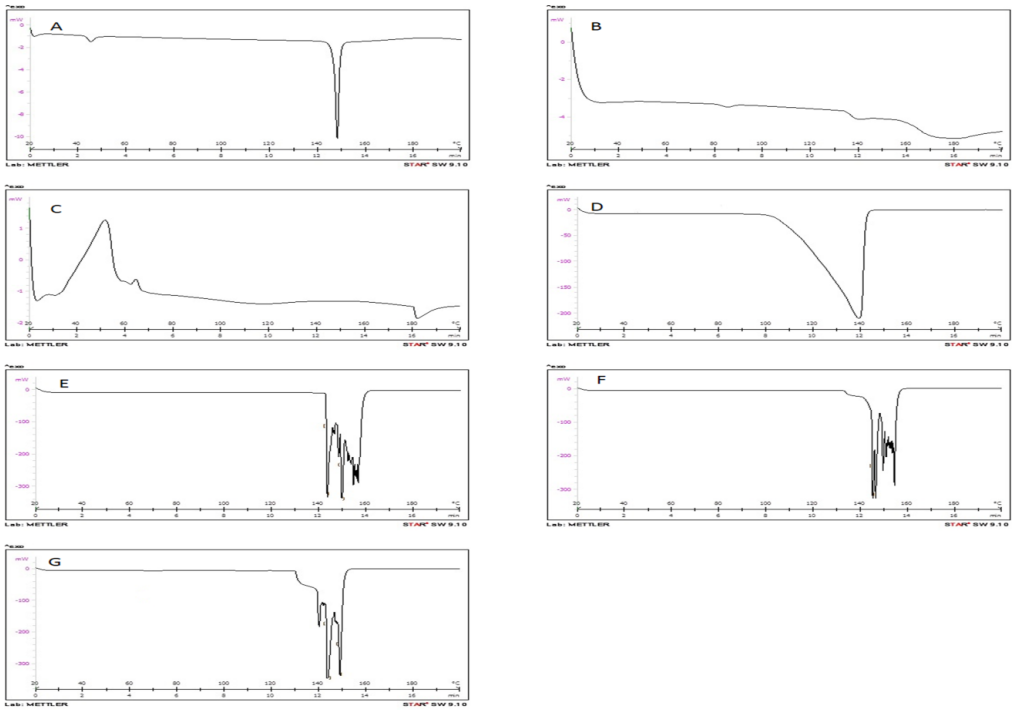


Figure 2. DSC thermogram of cholesterol (A), SPC (B), quercetin (C), F1 (D), F2 (E), F3 (F), F4 (G)

Table 3. Retention (%) and ratio of retention/penetration of quercetin from formulations (mean ± SD, n=3)

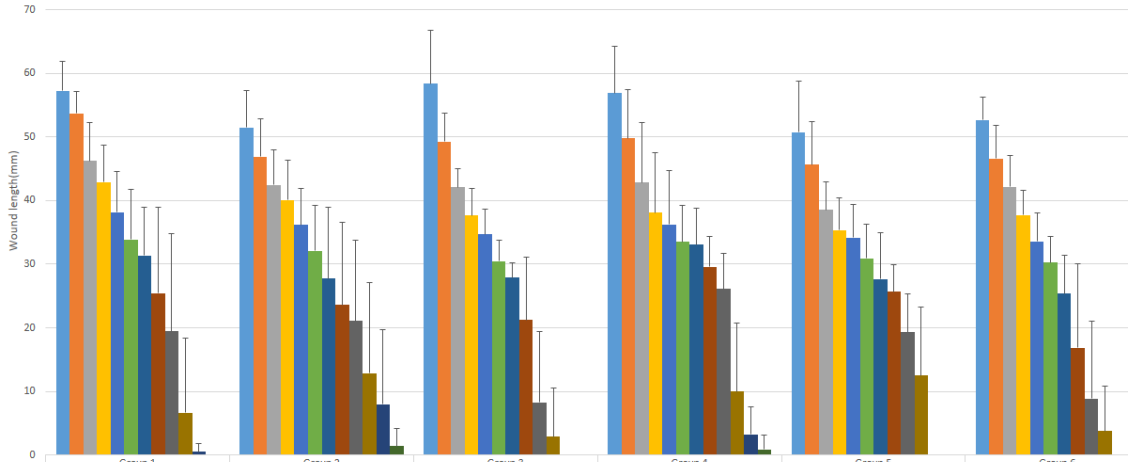
Formulation (quercetin, g/ml)	Retention (%)	Retention / Penetration
F2 (0.001)	51.41± 1.48	7.13 ± 0.34
F3 (0.002)	52.66 ± 0.96	8.98 ± 0.20
F4 (0.004)	46.13 ± 0.71	8.58 ± 0.48

systemic absorption in order to have the optimal effect on wound healing. The percentage of quercetin penetrated within the skin after 24 hours was calculated for QL formulations. The results are presented as percent of drug (Retention %) and permeation ratio (Retention / Penetration) in Table 3. Analysis of variance (ANOVA) showed that there is no significant difference between F2 and F3 formulations in retention (%) (P>0.05). Percentage of retention of F4 was significantly (P<0.05) less than that of other formulations. ANOVA analysis showed no significant differences among the three formulations in permeation ratio (P>0.05).

Animal study

Macroscopic analysis

As indicated in the procedure, length of the wound and the percentage of wound area were measured in different days (Fig 3). In different days of experiment period, photographs were taken from the upper surface of the wound, and evaluated to measure the length of the wound and the percentage of the affected area (Fig 4). For measuring the length of the wound, the full picture of the animal was used and to evaluate the surface of the wound, the images were cut in the same way for all the rats to minimize the error in the measurements. Recovery occurred after 28 days from the start of treatment for the groups receiving the QL formulation (Fig 4B., C, and D) and the group treated with the phenytoin 1% cream (Fig 4E). In the group receiving blank liposomes (group 1), there was also an apparent improvement, but, as shown in Fig 4A. at the end of day 28, there are still lesions in the cutaneous tissue. In a group of animals that had not



	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
day 0	57.23	51.48	58.32	56.89	50.71	52.63
day 3	53.68	46.87	49.27	49.75	45.66	46.58
day 5	46.26	42.47	42.07	42.79	38.55	42.13
day 7	42.92	40.06	37.64	38.11	35.32	37.71
day 9	38.14	36.19	34.68	36.24	34.14	33.57
day 11	33.91	32.13	30.49	33.5	30.93	30.23
day 13	31.39	27.74	27.87	33.09	27.68	25.35
day 15	25.44	23.62	21.24	29.58	25.63	16.85
day 17	19.44	21.08	8.28	26.14	19.31	8.9
day 19	6.64	12.91	2.89	10.01	12.49	3.75
day 23	0.49	7.99	0	3.24	0	0
day 28	0	1.4	0	0.87	0	0

Fig 3.(A) Wound length (mm) and (B) wound area percent of groups in different days (mean±SD)

received any substance from the beginning of the treatment (group 6, Fig 4F.), at the end of 28 days, there were still lesions in the skin. With periodic reviews of photos of different days in groups, it was concluded that the amelioration and rate of recovery in the group receiving F4 and the group receiving the phenytoin 1% cream is more than the other groups.

Analysis of variance analysis (ANOVA) regarding the comparison of the length and surface area of the wound among 6 groups did not show a significant difference during the 28-day treatment period (P>0.05). The results of the repeated measure of the intra-group regarding the length and surface area of the wound in 6 groups of treatments showed a significant difference among the mean in different days (P<0.05).

The results of the tensile strength are given in the Table 4. ANOVA analysis showed no significant differences among all of the groups (P>0.05).

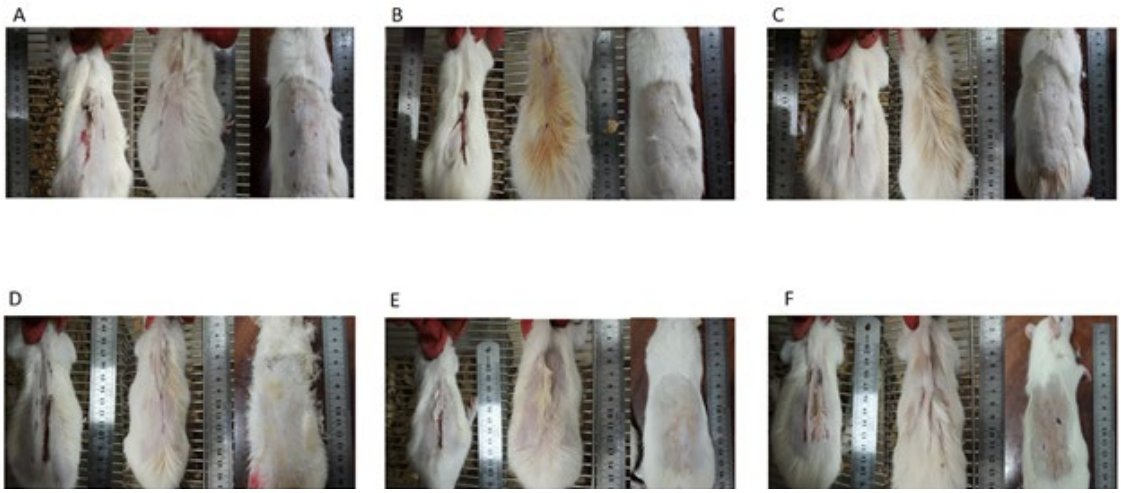


Fig 4.The left photograph is from day 0, the middle photo is from day 15, and right photo is from day 28 of the group receiving (A) liposomal blank (group 1), (B) F2 (group 2), (C) F3 (group 3), (D) F4 (group 4), (E) phenytoin cream 1% (group 5), and (F) no medication (group 6)

Table 4.Peak force (N), Elongation at break (%) and tensile strength (N/mm2) of all groups (mean ± SD, n=6)

Groups	Peak force (N)	Elongation at break (%)	Tensile strength (N/mm²)
1	56.41 ± 12.08	164.71 ± 35.34	0.125 ± 0.026
2	66.6 ±7.71	211.93 ± 95.69	0.148 ± 0.017
3	55.36 ±11.73	211.52 ± 62.53	0.123 ± 0.026
4	66.97 ±16.54	175.01 ± 16.71	0.148 ± 0.036
5	65.43 ±47.67	145.10 ± 15.21	0.145 ± 0.105
6	74.55 ±10.16	215.51 ± 51.31	0.165 ± 0.022

Histopathological assessment

In microscopic examination (Fig 5.), the sections prepared from the wound in different groups on day 28. In group without medication (group 6), some of the ulcers were still in the reproductive and inflammatory stage. The proliferated keratinocyte cells on the sides of the wound were not yet matched, and the wound surface was still present in the sacrum, containing cell residues and strands. In the dermis, inflammatory tissue with many inflammatory cells was obvious.

In the F2 formulation group (group 2), some of the lesions were similar to those in the untreated group (group 6) in the reproductive and inflammatory stage, and some were also in the stage of rebuilding of injury. In the lower part of the scars, a germanous tissue containing many vessels and inflammatory cells was obvious.

In the F3 treatment group (group 3), the surface of all ulcers was covered by keratinocyte cells and the germinal tissue contained vessels and a few inflammatory cells was observed in the

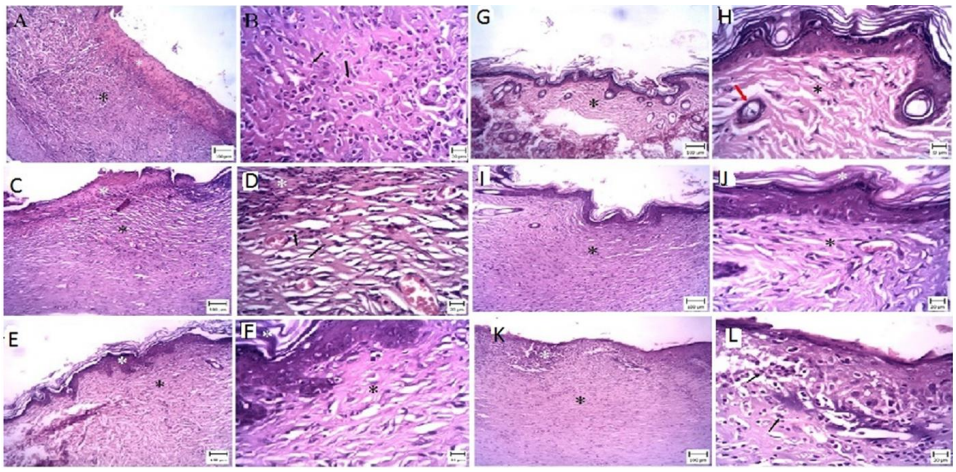


Fig 5. Rat skin, group without drug therapy (Group 6) (A, B). A: Look at the presence of a pink cuddle (white star) on the surface of the wound. It is also seen in the dermal tissue of the bud (black star). B: A germplasm with many inflammatory cells (arrows). (Staining of hematoxylin and eosin)

Rat skin, Group2 (F2) (C, D). C: Crust is seen on the surface of the wound (white star), and underneath it there is also a germinating tissue (black star). D: The debris of the degenerate cells (white star) and the lining of the juveniles with inflammatory cells (arrows).

Rat skin, Group 3 (F3) (E, F). E: The keratinocyte cells completely cover the wound surface (white star), and there are keratin layers on them. In the dermis part, the germinal tissue (black star) is visible. F: Consider the presence of keratin (white star) and stem tissue with a small number of inflammatory cells (black star)

Rat skin, Group 4 (F4) (G, H). G: The location of the wound is small and the keratinocyte cells completely cover the wound surface, and there are keratin layers on them. In the dermis part, the germinal tissue (black star) is visible. H: Consider the presence of keratin layers on the epidermal and germinal tissue with thick collagen bundles and the low number of inflammatory cells and fibroblasts (black star). The hair follicle (red arrow) is also developing in this area

Rat skin, Group of treatment with phenytoin cream1% (Group 5) (I, J). I: The keratinocyte cells completely cover the surface of the wound, and there are keratin layers on them. In the dermis part, the germinal tissue (black star) is visible. J: Consider the presence of keratin layers on the epidermal (white star) and the germinal tissue with thick collagen bundles and the low number of inflammatory cells and fibroblasts (black star)

Rat skin, Group with blank liposome (Group 1) (K, L). K: The keratinocyte cells do not fully cover the wound surface and there are no keratin layers on them. In the dermis part, the germinal tissue (black star) is visible. L: A budding tissue with a large number of inflammatory cells (arrows)

dermis. The keratinocytes on the surface of the wound began to produce keratin. The surface of the wounds in the F4 group (group 4) was well-improved and the wounds were re-established. In this group, collagen bundles were thickened in the stem tissue, and the number of cells and vessels were decreased and the similarity of this part with the adjacent healthy tissue was increased.

In the group treated with phenytoin 1% Cream (Group 5), the surface of all ulcers was covered by keratinocytes. The amount of vascularity and the number of inflammatory cells were decreased and the collagen bundles were thickened. In the group with liposomal blank medication (group 1), keratinocytes did not completely cover the surface of ulcers. There was also a high amount of vascularity and inflammatory cells in the tissue.

Trichrome Mason staining method was used to measure collagen level of tissue. In this method, collagen fibers are shown in blue (Fig 6.). The groups without drug therapy (group 6), the F2 formulation (group 2) and the non-drug liposomal group (group 1), had narrow collagen fibers. In the treatment group of F3 (group 3), F4 (group 4) and phenytoin (group 5), thick and regular blue collagen bundles were observed. This suggests that recovery has progressed in groups with thick collagen bundles, and the damaged tissue is similar to healthy tissue around.

DISCUSSION

In this study, fusion method was used to prepare liposome formulations, which is simple, efficient, and free of organic solvent such as chloroform. Using this method, homogeneous and high loading liposomes were prepared. In addition, fusion-made liposomes are viscous enough to be directly applied on the skin with no need to combine with other topical bases [34]. Particle size is a key feature in evaluating the properties of liposomes. Examining the particle size results shows that the particle size of all formulations is obtained in the appropriate range. Castangia et al., in a study conducted in 2014 on the properties of Quercetin and Curcumin nanovesicles in preventive and improving skin rejuvenation properties against invasive agents, have shown that by increasing the concentration of quercetin and curcumin in the nanovesicles the particle size also increases [35].

The amount of drug loading in the liposomes varied from 66.64 to 77.83%. Quercetin has a lipophilic nature, which can cause the substance to be placed into the liposomes bilayers and increase the amount of drug incorporation.

In various studies, the process of drug release from liposomes made with various phospholipids

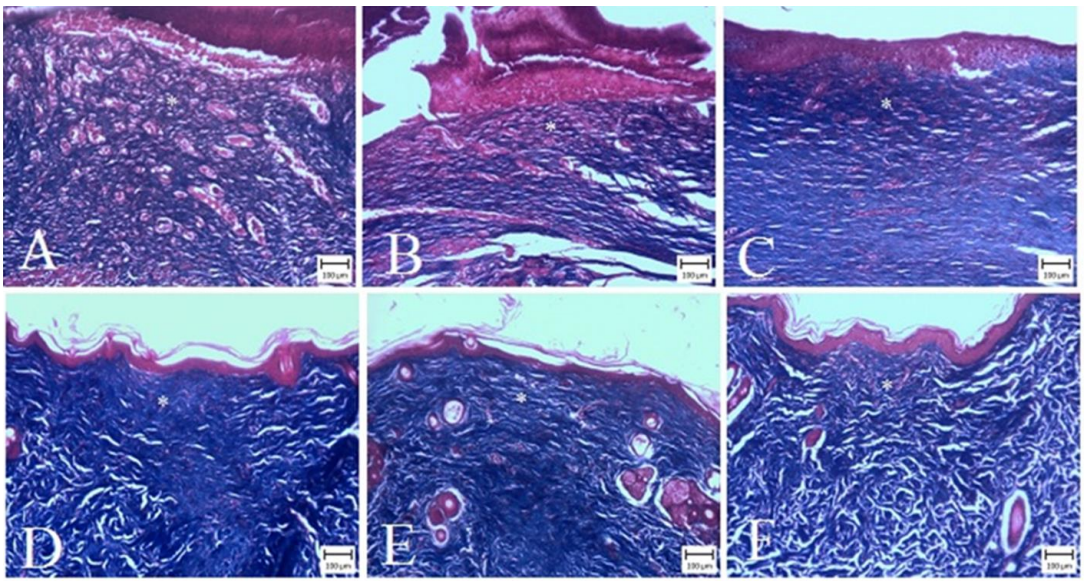


Fig 6. Rat skin, A: Untreated wound (group 6), B: Blank liposomal (Group 1), C: Formulation F2 (Group 2), D: Formulation F3 (Group 3), E: Formulation F4 (Group 4), F: Phenytoin cream1% (Group 5). Consider dense and thicker collagen bits in D, E, and F images compared to A, B, and C images (Trichrome Mason painting)

has been studied. The results of drug release from liposomal formulations showed that after 8 hours of initiation of the experiment, the percent of release reached its highest level. Considering that F4 formulations have the highest membrane release percent and the lowest loading among drug formulations, it can be concluded that the greater the amount of drug release was related to free drug that did not enter the liposome. The reason for the low release of drug through the skin rat membrane can be related to the lipophilic nature of quercetin and its tendency to the lipid phase. Liu et al., in a study to investigate the properties of quercetin solution and QL formulation, have finally shown that the liposomal formulation led to a slow release of the drug[36]. The results of drug permeability through the skin showed that the highest amount of drug remained in the membrane. This could indicate that systemic drug absorption is eliminated and most of the drug remains in the target tissue. The results indicate a lower penetration of F4 formulation in the skin, due to the lower amount of drug loading in the F4 formulation and the presence of free drug in the environment than other formulation. Encapsulation of the drug in suitable carriers can have positive effects on the drug's. An examination of the results of the residual drug in the skin indicates the presence of high doses of the drug within the skin, as the purpose of topical liposomal formulations is the minimum systemic absorption and higher drug retention in the target tissue to achieve the highest efficacy.

The use of liposomes in the pharmaceutical industry has significantly improved, especially by controlling and increasing the level of medication in the body and reducing side effects and reducing the dose[37].In general, liposomal formulation increases the penetration of the drug into the skin compared to the usual form of the drug. Also, drug delivery systems can be sustainable and targeted [38].

Different concentrations of phospholipid and unsaturated fatty acids in liposomes are one of the factors influencing the penetration of the drug into the skin. One of the reasons for using the liposome in the topical formulation of the drug is that the phospholipid in the liposome structure can penetrate the skin's lipid compounds and released into the subcutaneous tissue and by interaction with them, the membrane fluidity increases.

In the study of the stability of formulation, particle size changes of F2 and F3 formulations after 3 months of storage at the refrigerator temperature did not show a significant difference compared to the particle size of formulation immediately after preparation, which can be due to the presence of cholesterol in the formulation structure. Cholesterol in the structure of liposomes as a stabilizing agent prevents the accumulation and integration of vesicles, and thus can contribute to the physical stability of liposomes[31].Particle size variations for F4 formulation showed a significant difference with the time of liposomal production, which was still well within the range.

Stability studies also showed that there is no significant difference between encapsulation efficiency of F3 and F4 after 3 months storage and that of freshly prepared formulation. This could indicate that no structural change has been occurred in the liposomes. The amount of drug loading in F2 formulation after 3 months is significantly differ from that of initial time, which can be attributed to the breakdown of phospholipid layers and their re-formation during storage. It also includes some free drugs during re-formation.

Studies on the viscosity of formulations showed that viscosity was significantly different among all three formulations and the higher the concentration of quercetin in the formulation, the higher viscosity was obtained. In a study conducted by Fetih et al., The release and release rate of celecoxib drug in liposomal gel was reduced by increasing in the viscosity of the gel[39].The result of this study is contrary to the results of the research conducted by Fetih et al. that can be due to the fact that several factors including constituents of formulations and methods of preparation besides viscosity can impact on release and permeability.

In this study, compression injuries induced on the back of animals, liposomal formulations and phenytoin 1% cream were used twice a day for 28 days, and macroscopic and microscopic evaluation of the wounds and healing rate were evaluated in experimental and control groups. Wound healing is a multi-stage process that begins with scarring. The stages of this process include: inflammatory stage (stabilization of homeostasis and inflammation), reproductive phase (tissue granulation, wound closure and epithelization) and the rehabilitation phase (the formation of keratinocytes).

In the study of wound length and wound

percentage, it was shown that the recovery rate in the early days of formulation administration in all groups was significantly different from that in the last days of experiment. This means that in the early days, the recovery process was faster in the different groups of experiment, and after 14 days from the onset of the treatment when the relative improvement was occurred, the rate of recovery was reduced.

At the end of the 28-day period of treatment, apparent improvement was observed in all groups, but due to the apparent examination of the wounds of different groups during different days, it can be concluded that the rate of recovery in the groups treated with formulation contains the highest concentration of quercetin (F4), and the phenytoin 1% cream was higher than the rest of the groups.

In a study by Maha et al. on the effects of quercetin cream on improving skin scars, it was stated that after 4 weeks of starting the administration of formulations on hypertrophic wounds, improvement has been occurred in the groups treated with quercetin cream and placebo as well. The effect of quercetin over 4 weeks resulted in wound healing 40 percent more than in the placebo group, and after 8 weeks, improvement in the quercetin cream treated group was 20% higher than in the placebo group [40].

The surface area of the wound decreases as the experiment went on. The reason for this decrease is the presence of scarring and tissue deposition. The contraction phenomenon is due to the presence of myofibroblasts and their contractile nature that led to extend the inner layer of the epidermis and reduce the size of the wound [41].

Inflammation is the first response during the wound healing process and is considered as a defensive response, if this stage of the wound healing process is prolonged, it results in delayed wound healing [42]. The result of this study shows that the use of QL resulted in shortening of the period of this stage of the wound healing process, reduction of inflammatory cells at the site of the wound, and consequently, acceleration of wound healing. The amount of inflammatory cells in the group receiving the highest concentration of quercetin and phenytoin 1% cream is less than the other groups, due to the complete improvement in these two groups. In other groups, liposomal blank, QL (F2 and F3) and the group that did not receive any treatment, the inflammatory cell aggregation was clearly seen under the microscope.

A study by Kondo et al. has shown macrophages that attack the ulcer site in the first stage (the inflammation stage), play a major role in the formation of tissue granulation, which ultimately leads to healing of the tissue [43].The results of microscopic examination of hematoxylin-eosin staining showed that in the groups treated with either F4 or phenytoin cream of 1%, higher extent of granulated tissues and adult fibroblasts were evident rather than other groups, which is evidence for that the recovery process has taken place completely.

A study by Kyriakides et al. has shown that the organized parallel alignment of the collagen fibers is one of the features of the natural state of the skin, and the observation of this arrangement in the reconstructed tissue shows the proper repair process[44].The results of microscopic examination of hematoxylin-eosin staining showed that the presence of keratinized tissues on the surface of the wound in the group receiving the highest concentration of quercetin and the group treated with the phenytoin %1 cream was higher than other groups. This represents the final stages of wound healing and recovery.

CONCLUSION

According to the results obtained in this study, it can be concluded that administration of F4 formulation, as well as phenytoin %1 cream which is used in the clinic for the treatment of pressure ulcers or subcutaneous ulcers, result in wound healing. Due to its natural structure and minimal systemic absorption and side effects, this formulation can be a suitable candidate for the treatment of pressure ulcers.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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