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Liposome encapsulated curcumin in lysine-collagen hydrogel embedded with valsartan for treatment of diabetic wounds

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ABSTRACT

Impaired wound healing occurs due to factors such as diabetes, resulting in slow healing. The aim of this study is to develop and evaluate the wound healing potential of curcumin encapsulated liposomes in a lysine-collagen-hydrogel matrix embedded with valsartan for diabetic wounds. Formulations, CF1 (curcumin encapsulated liposomes in lysine–collagen hydrogel), CF2 (curcumin encapsulated liposomes in lysine-collagen hydrogel embedded with valsartan), and CF3 (valsartan loaded lysine-collagen hydrogel) were prepared and evaluated for physicochemical, histological, histomorphometric and wound healing properties. Formulation CF2 had the highest swelling ratio which was 89.2 ± 1.95%, while CF3 had the highest viscosity of 60000.00 ± 2.07 m Pas. Formulation CF2 showed the best wound closure, which was 100% by day seven, followed by CF1, CF3, Control and then diabetic wounds that were not treated. Formulation CF2 was found to be the most effective in promoting re-epithelization and angiogenesis. It can serve as an effective formulation for the treatment of diabetic wounds.

Keywords: liposome, hydrogel, wound healing, formulation, collagen

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INTRODUCTION

Diabetes is a condition that occurs when the pancreas does not produce enough insulin, or the body lacks the ability to utilize the insulin produced by the pancreas effectively[1]. This condition may lead to a consistent state of hyperglycaemia and, if poorly managed, may cause damage to the blood vessels and nerves[2]. Wounds that occur in people with poorly managed diabetes have a low chance of healing normally. The process of healing is impaired due to the hyperglycaemic state and the wound becomes chronic[3]. Chronic wounds emanating from poorly managed diabetes are an important primary public health issue. More than 430 million adults are likely to be affected by diabetic wounds within the next decade[4]. A diabetic wound can be described as an impaired wound characterized by hypoxia, impaired neovascularization, neuropathy and fibroblast abnormalities[4].

Diabetes has been identified as a leading cause of impaired wound healing[5]. Diabetic wounds are difficult to treat due to a variety of factors, which result in slow wound healing. The adverse microenvironment of a diabetic wound includes factors such as degenerative enzymes, alkaline pH and a complicated array of biochemical cues and processes that lead to a lack of progression through the primary phases of wound healing[6]. The impaired or chronic state of the wound is also caused by the high blood sugar levels, making it very unlikely that its entire process of angiogenesis will pass through all four structured phases of wound healing. Systemic factors that affect wound healing due to diabetes include sustained hyperglycaemia, peripheral neuropathy, and inflammation at the wound site[7].

The complicated process of tissue repair relies on the combined effect of cells, cytokines, enzymes and growth factors. In a diabetic wound, there is a lack of proper regulation necessary for wound healing, which leads to the wound into a chronic state[8]. Chronic diabetic wounds need an effective ultra-modern delivery system that will serve as an ideal wound treatment. This novel formulation should prevent infection, control the moisture level at the wound bed, facilitate the principal mechanisms involved in angiogenesis while stimulating wound closure, and finally, minimise scar formation[7].

Curcuma longa is a plant from which curcumin is gotten. It is a turmeric plant which belongs to a group of rhizomes[9]. The pathway through which curcumin enhances wound healing is through the localization of transforming growth factor β1 in the wound's microenvironment, coordination of collagen and reduction of reactive oxidative species. Curcumin is lipophilic, hence its de-

creased bioavailability and wound healing activity. Liposomes may be used for the encapsulation of hydrophobic drugs like curcumin to enhance their bioavailability and therapeutic activity[9].

The need for advancement of the curcumin-loaded liposome formulation may lead to the infusion of the loaded liposomes into a polymer hydrogel to improve the overall stability, efficacy, and dermal contact time of the formulation with the wound area. The development of a liposome-in-hydrogel delivery system will demonstrate improved hydrophilicity and controlled release of the loaded drug molecules curcumin, lysine and valsartan[10]. In this liposome-in-hydrogel delivery system, the surrounding polymer hydrogel provides a solid framework which serves as a mechanical cushion for the liposome bi-lipid membrane, enhancing its stability[11]. The delivery system provides an attenuated burst release effect via the liposome due to the mechanical cushioning effect of the hydrogel. It provides better muco-adhesion to the wound, higher tissue localisation, as well as efficient muco-penetration of loaded drug molecules into the wound microenvironment[12]. Research also indicates that the presence of lysine at the wound microenvironment may enhance wound healing, collagen will improve the tensile strength of the newly formed cells in the wound's microenvironment, while valsartan may be responsible for accelerated wound contraction, increased tensile strength, regulation of immune responses, as well as molecular and cellular processes. This makes this group of drug molecules an ideal choice for potentiating a synergistic wound healing effect[13,14,15]. The synergistic effect between these bioactive compounds presents a novel approach to diabetic wound management[16]. The choice of polymer used for the development of the hydrogel was based on its characteristic features that aid drug delivery at the wound site. Carbopol delivers maximum drug in an alkaline environment due to its greater swelling index at higher pH.[17]. The pH of a diabetic wound is slightly alkaline (6.95)[18]. Carbopol, an acrylic polymer and gelling agent, is safe for dermal applications and non-toxic[19].

Based on existing literature, little research has been carried out regarding synergistic interactions between bioactive molecules such as curcumin, lysine, valsartan, and collagen. In this study, curcumin-loaded liposomes in a lysine collagen hydrogel embedded with valsartan is formulated. This liposome-in-hydrogel based formulation stands out as a pioneer in its research space, as it provides a stable environment for the synergistic interaction between the therapeutic agents (curcumin, lysine, collagen and valsartan), while also providing sustained release of these agents. The formulation developed in this study could be used to enhance the healing of chronic diabetic wounds[20].

METHODOLOGY

Materials

The materials utilized for this study include are Phosphatidylcholine (Sigma-Aldrich Co., St. Louis®, MO, USA), Carbopol Ultrez (Surfachem, U.K), Valsartan (Merck, Germany), Curcumin (Sigma-Aldrich Co., St. Louis®, MO, USA), Phosphate buffer (Loba Chemie, Colaba Mumbai, India), Urethane (Sigma-Aldrich Co., St. Louis®, MO, USA), Methanol (Merck, Darmstadt, Germany), Cremophor (RH 40) (Macklin Biochemical, Shangai, China), Alloxan (Merck, Germany), Deionised water, Lysine (Sigma-Aldrich Co., St. Louis®, MO, USA), Collagen (**N**eocell, U.S.A), and Triethanolamine (Merck, New Jersey®, USA).

Development of curcumin-loaded liposomes

Liposomes were prepared according to 'thin film hydration method'[5]. Methanol (25 mL) was measured using a volumetric cylinder and poured into a round bottom flask. Curcumin and phosphatidylcholine (435 mg and 100 mg, respectively) were weighed using an analytical balance. The curcumin and phosphatidylcholine were dissolved in methanol in the flask, which was then attached to a rotary evaporator at 45°C. After 20 min, a lipid film is observed on the inner surface of a spherical flask. Phosphate buffer (pH 7.4) was prepared, and 25 mL of buffer was used to rehydrate the thin lipid film. The mixture was then sonicated for 15 min. Finally, the solution was vortexed for 10 min, and transferred into a 100 cm^3 transparent bottle. It was labelled and stored at 4°C. Liposomes were analysed in terms of shape, size, and surface morphology using Scanning electron microscopy[21].

Curcumin *in-vitro* drug release profile (Flow rate using Franz cell)

In vitro drug release study was performed with Franz diffusion cell. The diffusion fluid was a mixture of phosphate buffer (pH 7.4) and Cremophor RH40 (pH 7.0). The receptor compartment was filled with the diffusion fluid. A membrane filter was soaked in the diffusion fluid for 45 min, and then blotted on both sides. The membrane was fixed on the lower side of the donor compartment and fitted tightly with a ring. The Franz diffusion cell was fixed on a magnetic mixer and allowed to be stable at 37°C by warming. The donor compartment was filled with the curcumin-loaded liposomes. A stopwatch was started, and exactly 1 mL of sample was withdrawn from the diffusion fluid at intervals of 5, 10, 30, 60, 120, and 180 min. Then, 1 mL of fresh diffusion fluid was used to replace the fluid withdrawn from the receptor compartment of the Franz diffusion cell. Samples from the different time points were taken for UV analysis, and the experiment was performed in triplicates[17, 22].

Determination of encapsulation efficacy

Exactly 5 mL of the liposomal suspension was poured into a centrifuge tube and loaded onto a centrifuge, then allowed to centrifuge for 10 min at 400 rpm. The centrifuge tube was then removed, and the supernatant was discarded, while the loaded liposomes which had settled at the bottom were taken for UV analysis. Encapsulation efficacy was calculated using Equation (1). The experiment was performed in triplicate[23,24].

$$\text{Encapsulation Efficiency} = \frac{\text{TAC} - \text{NEAC}}{\text{TAC}} \; x \; 100 \quad \text{(Equation 1)}$$

TAC: Total amount of curcumin

NEAC: Non encapsulated amount of curcumin

Preparation of hydrogel formulations

Hydrogels were formulated by dissolving 8 g of Carbopol Ultrez in 0.4 L of distilled water, then left to soak overnight. Hydrogel cross-linking was carried out by adding three drops of triethanolamine, and the pH of the hydrogel was adjusted to pH 5.8 using sodium hydroxide or hydrochloric acid. The compositions of the different formulations are shown in Table 1. Curcumin-loaded liposomal formulation, 0.005 L, was incorporated into 0.4 L of hydrogel. Then, 0.005 g of lysine and 1g of collagen were added to the hydrogel to obtain formulation CF1. Formulation CF2 was prepared by adding 0.005 L of curcumin-loaded liposomal formulation into 0.4 L of hydrogel, then incorporating 0.004 g of valsartan, 5 mg of lysine and 1000 mg of collagen. Finally, formulation CF3 was prepared by adding 0.004 g of valsartan and 1000 mg of collagen into 400 cm^3 of polymer hydrogel. They were stored at 4°C[17,19].

Table 1. Formulations CF1-CF3 and their varying constituents

Ingredients	CF1	CF2	CF3
(2% w/v Carbopol) Polymer Hydrogel	0.4 L	0.4 L	0.4 L
Curcumin loaded liposomes	0.005 L	0.005 L	-
Lysine	0.005 g	0.005 g	-
Collagen	1 g	1 g	1 g
Valsartan	-	0.004 g	0.004 g

Physicochemical characteristics of hydrogel formulations CF1-CF3

Characterisation and pH evaluation of the hydrogel formulations CF1-CF3

Physical evaluation and pH determination of the formulations were carried out after preparation. The formulations were optically examined for consistency, homogeneity, and colour. The pH of the hydrogel formulations was measured using a pH meter (Mettler Toledo, Columbus USA in triplicate[25].

Rheology test

The rheological behaviour of the hydrogel formulations was determined my measuring their viscosity at 24 °C, at 20–100 rpm using Spindles 6.0 and 7.0 cone and plate viscometer (Brookfield Engineering Laboratories, Middleboro, USA). All measurements were performed in triplicate[25,26].

Swelling test

The extent of water absorbed by the hydrogel formulations was determined by incubating 0.1 g of dry thick hydrogel film in 50 cm^3 of phosphate-buffer saline (pH 7.4) at 37 °C. The dry weights of the formulations were denoted as Sa and equilibrium swelling weight as Sb. All measurements were carried out in triplicate[25].

The swelling ratio was expressed as:

$$\% \text{ Swelling ratio } = \frac{(Sb - Sa)}{Sa} x\ 100 \quad \text{(Equation 2)}$$

Stability studies

Stability tests were performed after the formulations CF1-CF3 were stored for one month at room temperature (23–24 °C). The appearance, texture properties and bio adhesiveness of the hydrogel were determined one day after preparation and during storage on days 3, 7, 14, 15, 30 and 60[25].

In-vivo wound healing studies

Twenty-four male Wistar rats, each weighing 380–420 g, were acquired at the beginning of the study. The rats were allowed to adapt to their new environment for one week and were housed individually in polypropylene cages. Ethical approval was obtained for this investigation with the approval number, CMUL/ACUREC/08/21/923. Twenty-four randomly selected rats were divided into two sets: diabetes-induced rats (n=20) and healthy rats (n=4). Both sets of animals were made to fast overnight. Diabetes was induced by

the intraperitoneal injection of a freshly prepared solution of alloxan (50 mg/kg) in 0.9% normal saline (pH 5.5). After seven days, blood sugar levels were tested using a glucometer (Accu-Check, Roche Diabetes Care Limited, U.S.A). Rats with blood sugar levels equal to or greater than 250 mg/dL were considered diabetic. All rats were anaesthetized intraperitoneally with urethane (0.03 cm^3/kg). The dorsal area of each Wistar rat was carefully shaved and cleaned with 70% ethanol. One excision wound was created on the upper back of each animal using a scalpel. Bioactive dressing containing formulations CF1, CF2 and CF3 were used to dress the diabetic wounds starting from day three, while untreated non-diabetic wounds were designated "control" and diabetic wounds not treated were designated "DNT". Photographs of the wound surface were taken, and wound closure was measured on days 1, 3, 7, and 14 post-treatment. The wound dressing was changed on days 3, 7 and 14, and wound size was measured using a calliper (Mitutoyo 500-196-30, Europe). Data were reported as percentage wound closure against time. The percentage of wound contraction was calculated using Equation 3[27].

$$\% \text{ wound closure} = \frac{A_0 - A_1}{A_0} \times 100 \quad \text{(Equation 3)}$$

A_0 = Wound size on day 0

A_1 = Wound size by day 3, 7, 14 and 21 after-treatment

Skin patch test

The hydrogel formulations (0.4 g) were applied to a shaved dorsal surface (3.0 cm^2) of three male Wistar rats. The skin appearance was visually examined for redness and swelling 1h after application[25].

Histological and histomorphometric examination

After the completion of the *in-vivo* wound studies, Wistar rats were euthanized. The healed wound sites were excised and fixed in 10% neutral buffered formalin. The tissues were immersed in alcohol then xylene. Embedding was carried out using paraffin wax. The tissues were stained with haematoxylin and eosin (H&E) and viewed under a microscope (Leica Microsystems microscope, Mannheim, Germany)[25].

Statistical analysis

The level of significant difference was considered if $p<0.05$. Statistical analyses were performed using Graph Pad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA)[28].

RESULTS and DISCUSSION

Size and morphology of curcumin-loaded liposomes

Liposomes were viewed under a scanning electron microscope, as seen in Figure 1. The liposomes were within the size range of 5 μm-10 μm in diameter. They were spherical with smooth surfaces, and also appeared stable. The large size of the liposomes enabled the encapsulation of a higher quantity of curcumin, which allowed for the release of more of curcumin locally at the wound area[29].

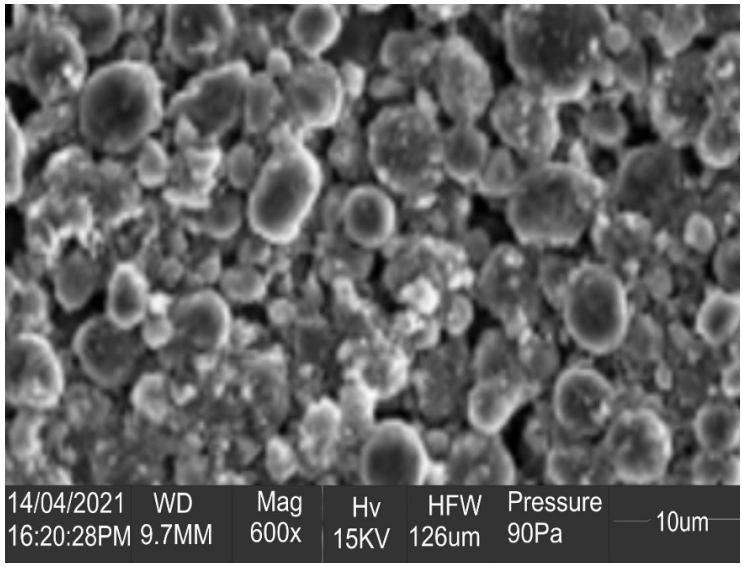

Figure 1. Scanning electron microscopic image of stable curcumin loaded liposomes

Encapsulation efficiency

The encapsulation efficiency of the curcumin-loaded liposomes was 99.934%, which is above 90%, indicating that the liposome loading of curcumin was optimal. This also implies that the liposomes remained stable with minimal leakage of curcumin. The stability of liposomes can be due to a couple of factors, such as the technique employed in its preparation, the type of phospholipid used, their size (which is influenced by the sonication time and frequency), and the storage conditions. Curcumin-loaded liposomes were prepared using a well-established technique (thin lipid film hydration), liposomes were also sonicated at a high frequency and an ideal time lapse. Also, liposomes were stored at 4°C immediately after preparation to prevent bilayer membrane disruption due to possible lipid hydrolysis[29].

In-vitro permeation rate (flux)

Flux illustrates a phenomenon in which a substance, in this case, drug molecule curcumin, appears to pass through a membrane selectively. Flux is also affected by the size, morphology, and encapsulation efficiency of the liposome formulation. A larger liposome size provides a larger of bi-lipid area to house the lipophilic drug curcumin. More efficient encapsulation of curcumin is associated with higher concentrations of curcumin per liposome and better flux, as more curcumin will be available to sip through the membrane. *In-vitro* permeability, also known as flux, as seen in Figure 2 was optimal (51.229 μg/cm²/h)[30].

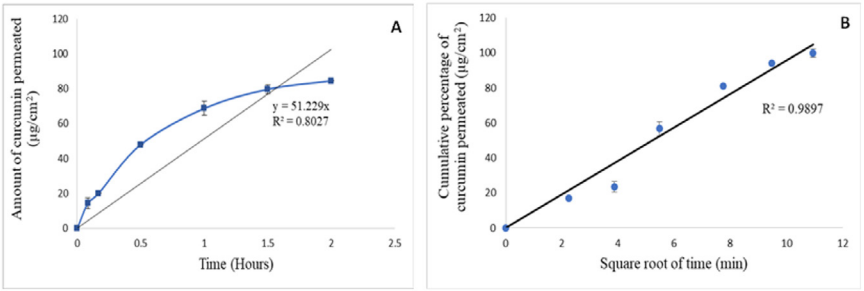

Figure 2. The flux of the optimized curcumin loaded liposomal formulation (A). The Higuchi plot of percentage cumulative of curcumin released from the optimized formulation against square root of time (B). Data are shown as mean ± standard deviation.

Figure 2B shows a plot of percentage cumulative drug release against the square root of time. This plot is that of the Higuchi release model. This model can help to understand the basic drug release system of the curcumin-loaded liposomes. Analysis of the release behaviour of the liposome formulations shows a controlled release of curcumin over a definite period. The release of curcumin from the liposome involved both dissolution and diffusion mechanisms. As a result, Higuchi release model can be used to fit the release of curcumin from liposomes[30].

Rheological evaluation

Rheology can be described as the science of flow and deformation of a material. It addresses the relationship between a given deformation and the stress response for a material such as hydrogel. Rheological techniques are commonly used to evaluate a material's viscosity and viscoelastic properties in relation to time, temperature, and shear.

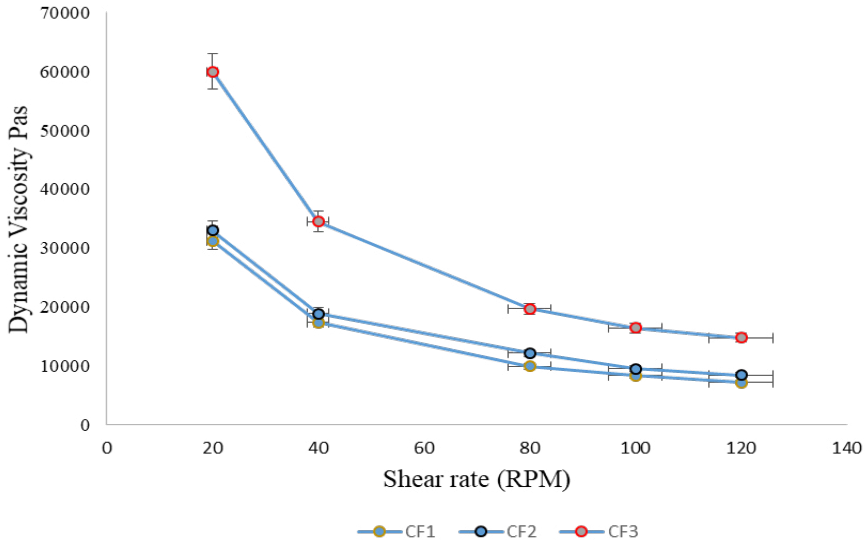

Figure 3. The rheological plot of dynamic viscosity in Pascal against shear rate in rotation per minute for formulations CF1-CF3. Data are shown as mean ± standard deviation.

In Figure 3, it can be deducted that there was a decrease in dynamic viscosity with an increase in shear rate, indicating that the flow resistance decreased on an increase in shear strain. This implies that the formulations are easily spread on topical application. All formulations showed a shear-thinning behaviour[31].

Table 2. Physicochemical properties of formulations, CF1, CF2, and CF3

Hydrogel Formulation	Dynamic Viscosity (20 rpm, PaS)	pH	Swelling Index %	Skin Irritancy
CF1	31250 ± 1.97***	5.8 ± 1.72*	85.7 ± 1.21***	Nil
CF2	32950 ± 1.01***	5.8 ± 1.11*	89.2 ± 1.95***	Nil
CF3	60000 ± 2.07***	5.8 ± 1.30*	81.4 ± 0.82***	Nil

Results are expressed as mean ± S.D (n=3). * Signifies $p<0.05$, ** signifies $p<0.01$, *** signifies $p<0.001$ with regard to significant differences.

As seen in Table 2, formulation CF3 had higher viscosity due to the absence of lysine, which tends to break hydrogel polymer chains. Also, the absence curcumin loaded liposomes contributed to its high viscosity, showing that the incorporation of curcumin loaded liposomes in the polymer hydrogel affected

the texture and consistency of the overall formulation. Formulations CF1 and CF2 had a more fluid like consistency and hence lower viscosity[32].

Physicochemical properties of formulations CF1-CF3

The formulations appeared stable, with no change in texture, smell, or visual appearance, and all formulations maintained their original texture and bio adhesiveness throughout the period of this study. There was no sign of redness or swelling one hour after application in all formulations on rat skin. Formulations CF1 and CF2 were translucent and pale orange with no odour, while formulation CF3 was opaque white in colour with no odour as well. The pH test was also carried out to ascertain that the formulation was dermatologically safe. The natural pH of the skin is 5.5-5.7, and the closeness of the formulations' pH (5.8) to that of the skin also indicates its dermal safety for application. A pH below 6.0 is suitable as it creates an environment to promote angiogenesis. This is because an acidic wound bed can inhibit microbial growth and maintain a microbe free wound bed, channelling it towards its expected healing pathway[33].

The swelling index gives an indication of the level of porosity and cross-linkage that occurs on a molecular and structural level in the hydrogel. The swelling index of a hydrogel is also affected by pH and temperature. This influences liposome uptake and release from the hydrogel. Formulation CF2 had the peak swelling index, while CF3 had the least. The higher the level of cross-linkage the lower the swelling index. The relationship between drug release rate and the level of cross-linkage of hydrogel matrix is direct: the higher the level of cross-linkage, the faster the drug release rate[34].

In-vivo wound healing studies

Angiogenesis is a normal biological reaction to tissue damage. However, wound healing is not a straightforward process as it involves complicated interactions between different cell types, cytokines, mediators, and the vascular system. Bleeding at the onset of an injury is reduced by a domino effect of instant constriction of capillaries and platelet accumulation. This is followed by an infiltration of various inflammatory cells. These inflammatory cells secrete a myriad of mediators and cytokines to enhance angiogenesis, thrombosis, and re-epithelialization, leading to wound contraction. In a diabetic wound, the chronic inflammatory response at the initial stage is sub-optimal and prolonged, so it becomes excessive at the latter stages of wound healing[35]. Angiogenesis is impaired by poorly controlled diabetes, and there is a marked presence of neuropathy with a very high risk of poly-microbial infection. All these factors lead to slow wound contraction and impaired wound healing in diabetic

wounds. Although inflammation is an important element needed to confine and eliminate bacterial contamination at the earlier stages of wound healing, excessive or prolonged inflammation towards the latter stages of wound healing may result in a chronic state. Excessive inflammation was observed in DNT, as wounds presented redness and swelling, indicating excessive inflammation at latter stages at the wound site. The pictorial representation in Figure 4 shows that diabetic wounds treated with formulation CF2 had attained complete wound closure by day 7 post-incision. This proves that the treatments enhanced the facilitation and progression of wound healing. Wounds treated with formulation CF3 also showed improved wound healing progression, though not as impressive as those treated with CF2. This may be due to the absence of a strong wound healing enhancing agent, curcumin. Curcumin interferes positively with every stage of wound healing. It enhances epithelisation, collagen infiltration, formation of new tissues and capillaries, healthy remodelling, and rapid wound contraction. Curcumin acts by recruiting M2-like macrophages into white adipose tissues. This leads to the production of anti-inflammatory cytokines that are important for response to the presence of foreign bodies. It then sufficiently lessens inflammation by the stimulation of a prototypical proinflammatory signalling pathway known as the NF-KB pathway at the final stages of wound healing.

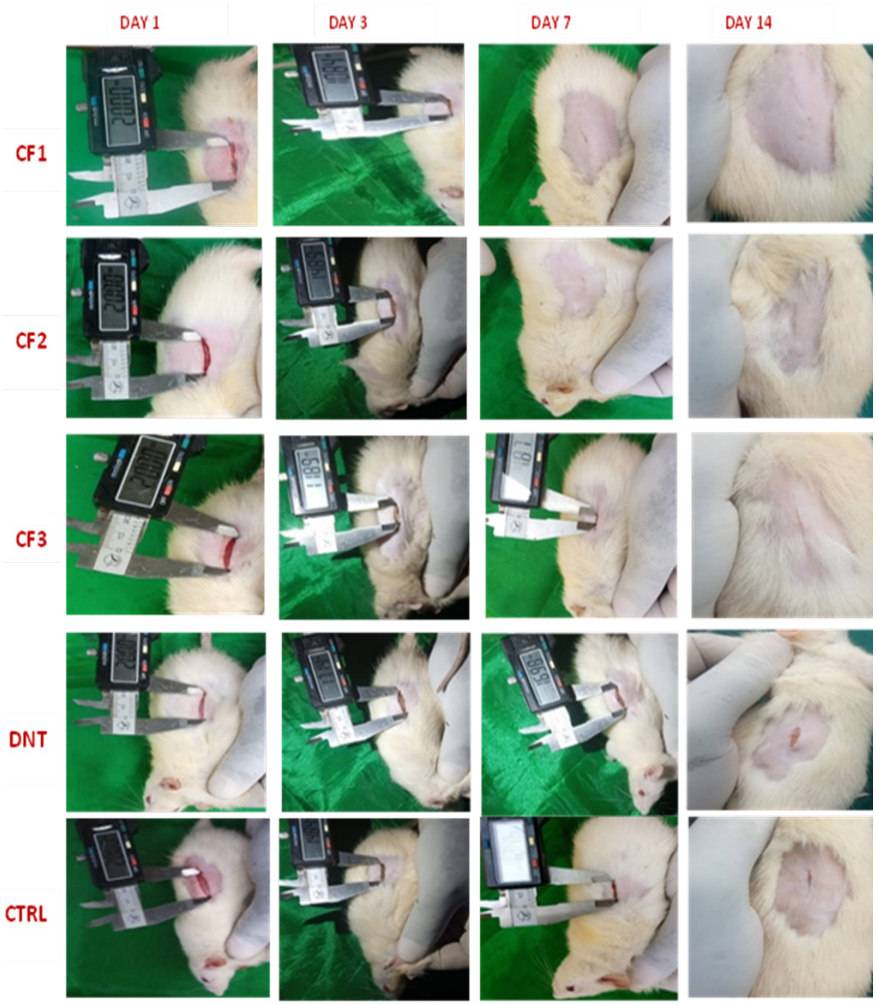

Figure 4. Pictorial representations of the contraction and re-epithelialization of wounds in rat groups treated with formulations (CF1, CF2, CF3), DNT (diabetic wound that were not treated with any formulation) and CTRL (non-diabetic wound that were not treated with any formulation).

The presence of curcumin along with other wound healing enhancing agents like lysine, collagen, and valsartan creates a synergistic effect responsible for facilitating wound healing. The absence of curcumin in formulation CF3 reflected in the slower wound healing progression in wounds of rats treated with these formulations. For all the treated wounds, complete closure was achieved by day 14 with no scarring observed. DNT healed with evidence of scars, indicating abnormal pattern of wound healing and remodelling. The control also

healed by day 14 with minimal scarification observed. Wound closure was rapid with no scars in chronic diabetic wounds treated with the developed formulations (CF1-CF3) compared to the control, which was not treated with any formulation. This indicates that tissue regeneration and re-epithelization rates were better in treated wounds. Complete re-epithelization and angiogenesis occurred within seven days for rats treated with formulation CF2, with regrowth of fur at the healed wound, as seen in Figure 4[36].

Relative wound size reduction and histological evaluation

Microscopic images of the haematoxylin and eosin staining of tissue sections from wound areas are shown in Figure 5A-E. All treated wounds showed the normal architecture of the skin, with the five layers of the epidermis intact, as well as the dermis[37]. DNT showed an abnormal tissue structure, showing clear perversion in the layers of the epidermis, the papillary dermis, and the reticular dermis (Figure 5E).

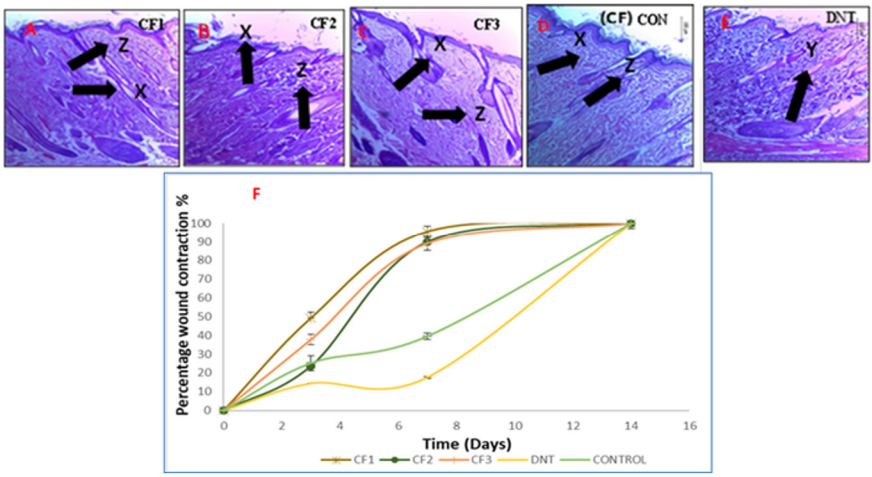

Figure 5. The microscopic images of the haematoxylin and eosin staining of tissue sections of wound areas of representative rats (A-E). The percentage relative wound size reduction from days 1 to day 14 (post-surgery) for chronic diabetic wounds (F). Data are shown as mean ± standard deviation.

The graph of percentage wound contraction against time, shown in 5F, showed that healing took place over a period of fourteen days and most of the wound contraction took place after the initial inflammation and proliferative phases[37]. All the rats used for the study survived through the post-operative process till euthanasia. Figures 5F shows wound healing curves similar to that of biological growth curves for all treated wounds. A different curve pattern was observed for DNT and control groups, proposing abnormal wound healing trajectory in these groups[38].

In Figure 5A-E, it can be seen that there is regrowth of the epidermis and presence of matured granulation tissue in diabetic wounds treated with formulation CF1, CF2, CF3, and the control. The arrow pointing to the spot marked X shows the wholly restructured epidermal and dermal layers, while spot Z shows the presence of newly formed connective tissues and capillaries, showing evidence of healthy wound healing. In Figure 5E, the spot Y shows a deformed tissue architecture for the DNT group, with abnormal layers of the epidermis and dermis, indicating poor wound healing and a lack of healthy regeneration of the dermal layers[39-41].

Histomorphometric analysis

Histomorphometry is described as the quantitative measurement of the shape or form of tissue[42]. It involves the quantitative analysis of parameters such as number of micro-vessels in the granulation tissue, percentage of collagen present in the granulation tissue, rates of re-epithelization, number of inflammatory tissues present and the thickness of the central region from the epidermis to dermis. These values are quantitative pointers to how well a wound has healed. The percentage re-epithelization rates show the level of migration of epithelial cells toward the wound bed for tissue repair, the epithelial cells achieve tissue repair through thick tissue formation[43]. The number of microvessels in granulation tissue shows the structural depth to which healing has occurred. Granulation tissue is basically composed of new connective tissue and micro blood vessels that form on the surface of a wound during the healing process. Granulation tissues progress from the base of the wound upwards to form the surface of a wound during the healing process[31]. Marked microvascular regeneration also indicates that proper neovascularization occurred in the vascular tissue at the wound bed[40]. The thickness of the central region of the epidermis and the dermis after wound healing shows how restructured the microenvironment of the wound bed is, whilst a high number of inflammatory cells in granulation tissue may mean that the wound is infected[41, 42].

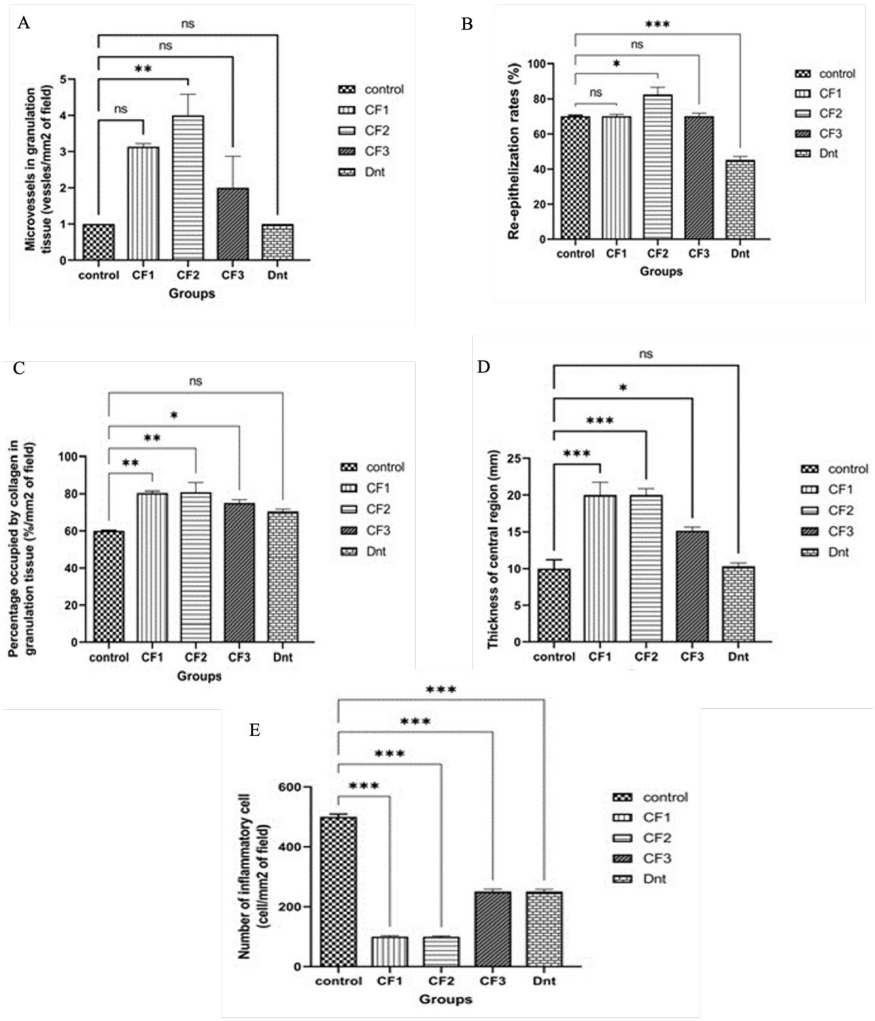

Figure 6. Histomorphometrical values for diabetic wound tissues fourteen days post wound incision (A - E). Data are shown as mean ± standard deviation. (*** means $p<0.001$, ** means p is between 0.001- 0.01, * means p is between 0.01 to 0.05, ad ns means $p \geq 0.05$).

Figures 6 (A-E) shows that the number of inflammatory cells were higher in the control group, indicating a likelihood of high microbial load at the wound site in the control group. The disparity between the control and other groups in terms of number of inflammatory cells is statistically significant. The re-epithelization rates obtained were highest for CF2 compared to the control and DNT. All wounds treated with formulations CF1, CF2, and CF3 showed re-epithelization rates above 70%, and they also contained higher collagen tissues. There was a clear and statistically significant difference between treatment group CF2 and

the control group in regard to the re-epithelization rates. The most therapeutically effective formulation, which enhanced rapid re-epithelization, contained curcumin, lysine, collagen, and valsartan (CF2). The formulations containing curcumin gave more rapid tissue regeneration[43]. This is primarily because the curcumin is favourably associated with the cellular events that occur in the inflammatory and proliferative phases[44]. Collagen is also known to contribute to the tissue tensile strength during angiogenesis at the wound bed[45]. Valsartan accelerates wound contraction, increases tensile strength of new tissue, regulates immune responses, as well as molecular and cellular processes in wound healing[13]. The number of micro vessels in the granulation tissue and the thickness of the central region of the epidermis to dermis indicate the depth of structural wound healing taking place as formation of micro vessels is necessary for vascular function at the wound site. Diabetic wounds treated with formulations CF1-CF3 showed a larger amount of micro vessels and a thicker central region of the epidermis and the dermis compared to the control and DNT (Figure 6). CF2 showed the highest level of significant difference when compared to the control in terms of number of micro vessels in tissue granulation and the thickness of the central region of the epidermis to dermis[36]. All formulations had hydrogel as base, however, curcumin-loaded liposome in lysine collagen hydrogel embedded with valsartan is preferred for management of diabetic chronic wounds due to its good swelling index, ideal viscosity, excellent in-vitro drug release, and encapsulation efficiency. Curcumin loaded liposome in lysine-collagen hydrogel embedded with valsartan demonstrated the peak wound contraction and was effective in promoting wound healing as it contained curcumin, collagen, lysine, and valsartan. The synergistic effect of these components had a clearly pronounced effect on angiogenesis at the wound site. Therefor this formulation can serve as an archetype for subsequent development as it portrays excellent therapeutic capabilities as a formulation for smart wound dressing for the management of diabetic chronic wounds.

STATEMENT OF ETHICS

Ethics approval with an ethical approval number CMUL/ACUREC/08/21/923 obtained and approved for animal studies via the Health Research Ethics Committee of College of Medicine, University of Lagos.

CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have any conflicting interests.

AUTHOR CONTRIBUTIONS

Cardoso-Daodu Ibilola and Ilomuanya Margaret: Conceptualization, Methodology, Software. Cardoso-Daodu Ibilola and Azubuike Chukwuemeka: Data Curation, Writing - Original Draft Preparation. Cardoso-Daodu Ibilola: Visualization, Investigation. Azubuike Chukwuemeka and Ilomuanya Margaret: Supervision. Cardoso-Daodu Ibilola: Validation. Cardoso-Daodu Ibilola, Azubuike Chukwuemeka and Ilomuanya Margaret: Writing - Reviewing and Editing.

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