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DEXTRAN-CHITOSAN-nTIO$_2$ TAILORED HYDROGELS (DEXCH-nTIO$_2$-HGS) AS VERSATILE TOOL IN EXPLORING THE POTENTIAL OF KAEMPFEROL FOR EFFECTIVE DIABETIC WOUND HEALING

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ABSTRACT

Grafts made from synthetic polymers perform poorly in wound healing applications. In these applications, dextran-chitosan based materials can be produced and fabricated to present a novel scaffold for wound healing engineering. The aim of this study was to evaluate *in-vitro* the mechanical properties of a novel dextran-chitosan based hydrogel formulation to examine its potential for this scaffold. Kaempferol (KMF) (obtained from leaves of *Ipomea carnea* Jacq) loaded hydrogel (HGs) was prepared using dextran-chitosan (Dex-CH) and PEG as a cross linking agent with nano titanium dioxide (nTiO$_2$). Subsequently Dex and CH solution were blended together to form a homogeneous DexCH binary blend mixture with the PEG as crosslinker and characterized for various paramters like AFM (Atomic Force Microscopy), mechanical (tensile) strength of hydrogel, swelling property, entrapment efficiency and drug release. The role of hydrogel was assessed by wound healing activity tested beside wound contraction, collagen content, dried granuloma weights and antioxidant property. Percent entrapment efficiency of optimized hydrogel found to be 89.36 ± 1.19%. KMF loaded DexCH- nTiO$_2$-HGs were able to release 95.36%, KMF in 24 hrs. The level of superoxide dismutase (SOD) and catalase were found increased significantly in granuloma tissue of KMF treated group. The significant wound healing effect in normal wound tissues as well as diabetic with higher antioxidant activity founded in KMF-DexCH-nTiO$_2$-HGs. Prepared hydrogel formulations (KMF loaded DexCH- nTiO$_2$-HGs) were proved to be highly suitable on grounds of moist nature, biocompatibility, biodegradability and antioxidant effectiveness.

Keywords: Hydrogels, Kaempferol, *Ipomea carnea*, nTiO$_2$, Wound healing, Antioxidant.

1. INTRODUCTION

Wounds are corporeal harm that results from infringement or opening of the skin. The practical condition and refurbishment of interrupted skin is crucial for appropriate wounds healing. It is fabricated with the integrated reaction of numerous tissue categories to injury. Wound healing is an intricate multifactorial and collective biological series which consist of inflammatory, proliferation and remodeling phases [1]. Inflammation phase employ to set up the homeostasis and inflam-mation; proliferative phase pursue with granulation, contraction and re-epithelialization. In diabetes pathic collagen metabolism is considered as a chief factor in delayed wound healing. Potential occlusion of capillary vessels with injured endothelial as well as hyperglycemia-induced leukocyte dysfunction, decreased chemotaxis and phagocytosis results in impaired wound healing, which thicken the basement membrane of the arterioles and capillaries in diabetic circumstance. It is frequently seen in individuals with diabetes, resulting in an immature wound healing and pushy ulcer formation [2]. Difficulty occurs as consequences of elevated glucose, impaired glucose tolerance and protein glycation [3]. In a while increase into deeper ulcers encourage complexities such as infection which takes place repeatedly, this need limitation of infection to the fundamental tissue, and delay in heal. On the other hand, the presence of high glucose level also changes to granulation tissue was lacking in collagen, cellular morphology, abnormal differentiation of keratinocytes, and decreased proliferation [4].

Ipomea carnea Jacq. family convolvulaceae, is a folk medicine used to treat of skin disease. A metal complexes prepared from the Leaves of *I. Carnea* posses antimicrobial and antifungal activity [5]. Traditionally the leaves of *I. carnea* externally applied on wounds and cuts to get quick relief [6], free radical scavenging, wound healing, [7] anti-inflammatory and antioxidant activity

[8], as well as used for laxative and mild purgative [9]. Leafy latex of *I. carnea* are already reported about the presence of flavonoid and evaluated for wound healing activity [10]. Plant flavonoids benefited the healing process by modulating the concentrations of reactive oxygen species by activating the platelets, neutrophils, macrophages, lymphocytes and fibroblasts at different time points of the healing process [11]. Kaempferol (KMF) is a natural flavonol, which is one of category of a flavonoid, originate in a diversity of natural herbs and herbs derived foods.

Various types of preprations are used for potential wound remedial like ointment, creams, nanoparticles, patches, wound dressings and in recent times especially admired are hydrogels (HGs).

KMF has biomedical relevance particularly for anti-proliferative, antiviral, antioxidant, antibacterial [12] wound healing and anti-inflammatory action [13]. A research accounted that administration of kaempferol to streptozotocin-induced diabetic animals (rats) revealed hypolipidimic. The metabolic changes of glycoprotein regressed towards normal stage when treated with kaempferol in diabetic rats and sialic acid extensively improved in plasma, liver and heart, whereas diminished in kidney of streptozocine stimulated diabetic [14]. A study has accounted that administration of kaempferol has potential to restore unbalance activity of membraine-bound ATPases in STZ-induced rats. Earlier, in an *in vitro* study, it was revealed that kaempferol ameliorates hyperglycemia by improving insulin-stimulated glucose uptake in adipocytes [15]. Kaempferol also achieved a favorable function in diabetes by avoiding oxidative damage in pancreatic β cells [16]. In contrast, the restriction of KMF is its poor water solubility and low bioavailability [17]. Accordingly, there is a thrust to develop innovative formulations or drug delivery system to overcome the bioavailability concerns related with KMF. Solubility and dissolution rate problems can be resolved by employing convinced practices like; particle size reduction, enhancement in wettability/porosity etc. There are numerous formulations for KMF are accounted by researchers such as nanocarriers, PLGA and sustainable lecithin/chitosan nanoparticles, polymeric micelles, and lipid nanocapsules and floating release [18, 19].

Hydrogel (HGs) is the networks of three-dimensional polymer, hydrophilic structure which get swollen in the presence of large quantity of solvent. The capabilities of hydrogels to absorb water arise from hydrophilic functional groups attached to the polymeric backbone, as their conflict to dissolution arises from cross-links amongst network chains. The HGs have rubbery soft texture and flexible, resembles with human tissues having considerable conflict as wound relevance. Hydrogel had gained significant attraction owing to their successful applications in tissue engineering, drug delivery [20, 21] as well, these systems also simplified activities required for wound healing, cell infiltration and retention of viability.

Dextran (Dex), a polymer (polysaccharide) is hydrophilic, harmless, biodegradable, biocompatible, water soluble, inert in biological system and highly hydrophilic consisting of glucose moiety along with hardly influenced the cell capability. Dextran hydrogels encourage rapid, efficient neovascularization, better biocompatibility and integration with the cells [22] this accelerates the mobilization of endothelial cells to the wound area, facilitating immediate neovascularization after a 5-7 days of medication management [23]. Dextran and its derivatives are used as plasma expanders, bone rejuvenation promoters in addition to skin and subcutaneous filling. Consequently, dextrans are the most competent candidate for design of hydrogels capable to manage the release of both minute particle and protein drugs [24]. Chemical hydrogels of dextran are arranged via chemical crosslinking of preformed physical networks, to obtain slower drug release, and improvement their mechanical properties.

Chitosan is a natural cationic copolymer with well deal of benefit for hydrogel structures owing to their well prominent biocompatibility and low toxicity [25]. Chitosan has capability of degradation via human enzymes which result in biocompatibility and biodegradability with hydrophilic nature. It performs for developing diffusion by opening epithelial tight-junctions along with positive charges at physiological pH, this enhance retention at the place of application because chitosan exerts the bioadhesive property. Additionally chitosan supports for wound healing and also works against bacterial infection [26].

Through permanent covalent crosslinked chitosan, chemical hydrogels are formed in current work. Hydrogel possess suitable mechanical and physical assets with physiological atmosphere to make possible cell adhesion, production of cell as well as avert secondary infection. Consequently hydrogels are prominent beneficial since they are capable to prevent hydrolysis, they offer sufficient gaseous exchange and absorb injury fluids. A huge variety of polysaccharides owing to their tremendous gel-forming possessions, biodegradability as

well as biocompatibility, like chitosan, hyaluronic acid, dextran, alginate and heparin formulate use as hydrogels for tissue engineering.

To accomplish the property of wound healing, $nTiO_2$ was preferred because it is hold antibacterial action and previously it is used to preparation of several cosmetic materials, additionally, titanium ions released from TiO_2 can increase keratinocyte movement towards the wounded area, consequently encouraging its healing [27]. It is stated in earlier researches that in the size range less than 100 nm and at the suitable concentration, $nTiO_2$ acquires effective antibacterial action due to its photocatalytic possessions with no harmful consequences on normal tissues [28]. Titanium dioxide (TiO2) particles are categorized as biologically inert in human beings because titanium reveal inferior toxicity than other oxides of nanometal [29]. However the toxicity of nanotitanium be able to work beside redundant bacterial growth [30].

Owing to the amorphous arrangement of the dextran-chitosan-nano titanium dioxide (Dex-CH- $nTiO_2$) hydrogel (HGs) be capable of acquire water up to 99% of own [31]. The column chromatography and spectroscopic technique are employed to separate kaempferol from ethanol extracts of *Ipomea carnea* leaves. This study summarized to design, characterize and investigate, dextran-chitosan-$nTiO_2$ polymer fabricated by kaempferol loaded HGs for antioxidant and chronic diabetic wound healing assets.

2. MATERIAL AND METHODS

2.1. Material

Ethyl acetate, petroleum ether, silica, benzene, chloroform, DMSO (Dimethyl sulfoxide), EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and PEG (Poly ethylene glycol) and EDTA were supplied from HiMedia Lab, Mumbai, India, standard streptozotocin and Methanol has been purchased from Merck Ltd, New Delhi, India. Dextran, chitosan and $nTiO_2$ were obtained by Sigma-Aldrich Co. (USA).

2.2. Methods

2.2.1. Extraction and isolation of kaempferol

The dry leaves (2.0 Kg) of *I. carnea* powdered in electrical grinder, and stored in an airtight brown bottle at 5°C until further employ. Later carry out for extraction and defatted with petroleum ether (60-80°C) pursued with ethanol (95%) for 72 hrs. Solvent recovered by distillation and residual solvent was distant under vacuum. The dried ethanolic extract treated with chloroform repeatedely to obtain chloroform soluble fraction was dissolved in methanol (MeOH) in a separating funnel repeatedly. MeOH *I. carnea* fraction (ICF) was desiccated under vacuum produced (1.8 % w/w) a greenish powdered resultant which were examined for existence of flavonoids.

The ICF was chromatographed using column chromatography using suspending silica (60-120 mesh; Merck) bed for 24 hrs in benzene. Further, slurry was loaded in flash glass chromatographic column (35 cm × 1.5 cm) and bed (diameter 28 cm × 1.5 cm) was permitted to settle. At about 2.0 g prepared sample transfer in a chromatographic column of ICF fraction. The elution of filtrate was chromatographed using the ethyl acetate and 20 eluates of 5 mL of each were collected [32]. Each eluted fractions prepared for TLC pattern performed using through the solvent system, Benzene: Chloroform: Methanol (14:14:2). The fraction of 10 to 20 was referred as compound 1 since solitary one spot experiential in the TLC pattern. Examined through, the spectroscopic analysis for re-crystallized compounds (0.72 mg) in methanol. The filtrate of isolated pure compound was collected and characterized by UV, infra red (IR), proton nuclear magnetic resonance (1H-NMR), and mass spectroscopy (MS). UV analysis was performed on a double-beam Chemito Instruments Pvt. Ltd UV 2600 (US) after methanolic liquification. IR spectra were traced on a spectrum 100 FT-IR spectrometer (Cary-630 FTIR, Agilent Technologies USA). The ^{1}HNMR study through a FT-NMR spectrometer 400 MHz (Bruker) using deuterated chloroform ($CDCl_3$) as solvent and TMS (tetramethylsilane) as internal standard, ESI-MS (Mass spectroscopy) spectrum was performed at 70 eV on a Water MSD Ion Trap XCT instrument (Japan).

2.2.2. Preparation of Chitosan-PEG-Dextran-$nTiO_2$ (Dex-CH-$nTiO_2$-HGs)

In 10 ml of double distilled water, the 30 mg chitosan polymer was dissolved and activation of chitosan was performed by mixing of 250 mg of EDAC, and then fivefold excess 250 mg of bi-functional PEG (COOH-PEG-NH_2) was added with incessant mixing for 2 hr and sonicated for 20 sec, for development of triggered polymeric mixture of chitosan-PEG-COOH dispersion. Kaempferol (0.25g) was dissolved into dextran-Chitosan-PEG. Amino-dextran (30 mg) (Amino dextran were synthesized as per method reported by Zalipsky), was dissolved in 100mL of distilled water and added to chitosan-PEG-COOH dispersion under constant stirring [33] and added the dry weight of 0.1% $nTiO_2$ earlier

sonicated ultrasonic homogenizer () for 1 hr and dispersed on the matrix gel dispersion and mix with magnetic stirrer (Remi, Mumbai, India) for 10h. The reaction mixture was comprehensively dialyzed (DiaEasy™, 12-14 kDa MWCO the capacity of 250 µl) to take apart Dex-PEG-CH- $nTiO_2$ from untreated Dextran, chitosan and $nTiO_2$. The achieved polymer named DexCH-$nTiO_2$ HGs was desiccated in 'Tanco' vacuum lyophilizer (PG-302). Characterization and identification of Kaempferol macromolecule linking polymers, Dex-CH-$nTiO_2$ -HGs was performed by Nuclear Magnetic Resonance (NMR, Bruker DRX, USA) operated at300 MHz and FTIR (FT-IR spectro-photometer Cary- 630 FTIR, Agilent Technologies USA) spectroscopy of DexCH-$nTiO_2$-HGs was performed at, after dissolution in DMSO.

2.2.3. In vitro Characterization

2.2.3.1. Hydrogel Imaging by Atomic Force Microscopic (AFM)

The structure of surface texture, height (topography) and shape, stiffness of DexCH-$nTiO_2$-HGs network were characterized through AIST-NT Smart SPM 1000, CA atomic force microscope (AFM) in fully hydrated sample. AFM of the HGs was executed by stiff substrate in tapping mode to illustrate the surface roughness which is observed as one of the essential surface assets that acts a significant function in membrane permeability and detestable conduct.

2.2.3.2. Measurement water absorption ratio

The swelling ability (water absorption ratio) was carried out by immersing in SWF (simulated wound fluid) in pH 7.5 an accurate weighed quantity of dry sample at a given time interval. The swollen sample was removed from water, and filter paper was employed to dehydrate the excess liquid after a definite time. Repeated the similar testing for an average of three times and calculated as the degree of water absorption ratio by following formula [34]

$$W(\%) = [\frac{m - m_0}{m}] \times 100$$

Where in formula m_0 and m are the weights of wet and dry hydrogel respectively and

W is the water content swelling (Absorption) ratio.

2.2.3.3. Tensile (Stress strain) test of hydrogel

The eXpert 7601 USA, with eP2 Digital Controller, peculiar roller grips, the tensile test tester were used for the estimation of breaking elongation and tensile force of hydrogels. The hydrogel membranes were cut into a specific cross section area (3.5 × 1.5 mm) in dumbbell shape and clamped vertically in stress-strain tester. The mechanical analysis (Stress strain Test) was achieved at a stretching rate of 0.170mm/s with 35 mm of gauge length [35]. The thickness of the hydrogel membrane was measured with a digital caliper (Phenovo IP 67, Hongkong) before examination until failure of stretch.

2.2.3.4. Determination Drug Entrapment

The pre-weighed section of absolutely dehydrated HGs of drug (KMF) entrapped for each gram of the hydrogel in 25ml of aqueous drug solution. The dry sample was placed in aqueous drug solution which having different drug concentration was prepared. For the symmetry loading its equilibrated for a period of 24 hrs and further, examination was achieved through the concentration of entrapped drug which was examine with HPLC (Agilent Technologies, 1220 infinity LS, UK) method. The HPLC study was completed through variable wavelength detector and a C18 column (zorbax 5µ, 250 × 4.60 mm) was used for the determination of sample at 265 nm. The mobile phase was water: methanol (95:5 v/v) were used with 1 mL/min flow rate at 25°C [36].

The following equation was used to calculate the concentration of drug entrapped per gram of HGs:

$$\text{Drug entrapment} = \frac{\text{Initial drug content in solution - Final drug content in solution}}{\text{Weight of dry sample}} \mu g/g$$

2.2.3.5. Drug release

The physiological fluid at about 25 ml was taken and at 37°C temperature, added the pre-weighed amount of drug loaded HGs. The 0.5ml of samples was taken out at 1-8, 10, 12, and 24 hours. The analysis of the amount of drug release at different time duration, were performed by the chromatography analysis. For the drug solution of known quantity the calibration curve was made in suitable range for the estimation of the concentration of released drug [36].

2.3. *In-vivo* wound healing study

2.3.1. Animals grouping

The inbred house pathogen free eighteen male wistar rats weighing 150-250gm adapted attained to laboratory hygienic environment for 10 days early were used in this testing. The rats were housed in microisolation house conditions with of 27±2°C temperature with controlled 45-55% of humidity. Animal were fed standard pellet diet (Lipton, India), and water *ad libitum* [37]. Approval from the Institutional Animal Ethical Committee was taken prior to the *In vivo* study (Registration No.

1471/PO/a/11/CPCSEA, India). The three groups of animals are divided for each model which is having of six animals in every group. Whereas the control group given vehicle (Simple DexCH- $nTiO_2$-HGs), test group received KMF loaded DexCH-$nTiO_2$-HGs and reference group applied marketed formulation, povidone iodine ointment USP 10% brand 'Povilet' (Altova Healthcare Limited Mahim West, Mumbai, Maharashtra). By the topically are treatments were applied.

2.3.2. Induction of Diabetic wound

Diabetic condition induced by the single dose of streptozotocin (70mg/kg) for six weeks prior in saline, injected IP (intraperitoneally) in experimental animal after overnight fasting, freshly prepared in 1 ml of phosphate buffer (pH 5.5) [38]. The fasting blood glucose levels of rats go beyond to 250 mg/dL (13.9 mmol/dL) and blood was drawn from the tail vein were taken for diabetic model. The blood glucose level predicted through the, (Biosen C-Line Glucose) glucometer, within three consecutive days to rats. On dorsum of all rats, which is previously rinsing, cleaning and shaved, having elevated blood glucose (more than 140 mg/dL) a circular excision wound was induced. The blood glucose level was noticed at the time of wounds formation and after receiving the treatment. In the diabetic wound model, percent of wound contraction, antioxidant level and histopathology study were carried out.

2.3.2.1. Incision wound model

Every experimental rat was anaesthetized previous to injury formation and a paravertebral 1 cm extended cut was prepared at dorsal portion of animals. The both edges kept jointly and sutured with black silk surgical thread (No. 000) and a curved needle (No. 11). No local or systemic antimicrobials are applied throughout the test. The incessant threads on both injury ends were constricted for superior closure of the injury [39]. All groups are medicated similar as in excision model. Subsequent to stitching, injury is missing naked and all test models be superficially practical every day up to 9 days. The sutures were detached on the day 9, when wounds were healed systematically and tensile strength of treated lesion skin was calculated by means of tensiometer [40].

2.3.3. Induction of Dead space wound

In dead space wound model the determination of granuloma tissue formation was done for study of estimation of biochemical parameters and dry granulation weight. Animals were anaesthetized by ketamine before and a wound was created by subcutaneously implanting of two polypropylene tube ($0.5\times2.0cm^2$), in the each side of dorsolumbar region of back, in all animals involve in this model. Around implanted tube the granuloma tissues were formed, which was cut apart upto 10^{th} post-wounding day carefully. The piece of dry granuloma tissue was collected from each tube to get the constant weight and weighed was determined and a part was stored in 10% formalin for the biochemical assay [41].

2.4. Wound healing evaluation

2.4.1. Wound closure time for excision wound

Wound contraction is the closure development to reduce the rate of uncured part. Consequently, the rapid time of wound closer furnished the enhanced worth of drug in the first two week. The percent of wound contraction of an excision wound was studied by tracing a transparent paper in every alternate day after creation the wound, until complete healing and expressed in percentage of healed wound area. The epithelialization time was measured from preliminary day [42]. The percentage wound contraction was calculated as given below formula:

$$\text{Percent wound contraction} = \frac{\text{healed area}}{\text{total area}} X100$$

2.4.2. Protein estimation and granuloma weight

On the post wounding days 18^{th} the protein content of skin tissues were determined by method reported by Lowry [43]. The tissue lysate was treated with a mixture of sodium tartrate, copper sulphate and sodium carbonate. The mixture was left to stand for 10 minutes and then treated with Folin-Ciocalteau reagent which gives a bluish color in 20-30 minutes. The absorbance was taken at 650 nm using Spectrophotometer.

2.4.3. Hydroxyproline measurement

Wound tissues were analyzed on 18^{th} day for hydroxyproline content which is a basic constituent of collagen. Tissues were dried at 60-70°C up to constant weight and samples were hydrolyzed with 6 N HCl for 4 h at 130°C. The hydrolysate was neutralized (pH 7) then subjected to Chloramine-T oxidation for 20 minutes. The colored adduct formed with Ehrlich reagent at 60°C through terminating the reaction by adding 0.4 M perchloric acid [44] and observed at 557 nm in UV spectrophotometer. Standard hydroxyproline was also runned and values reported as µg/mg dry weight of tissue.

2.4.4. Antioxidants assay in granuloma tissues

The granuloma tissues were determined for antioxidants assay by granulation tissue homogenates. By the collapsing of H_2O_2, catalase was determined subsequent by the process of [45]. Superoxide dismutase (SOD) evaluation was performed by the process of [46] mixing of homogenate tissue supernatant to buffer medium with EDTA in the enzyme. By the method of Moron [47] Reduced glutathione (GSH) level was estimated by the capability of GSH to reduce DTNB. After 5 min the addition of supernatant with complete homogenate tissue mixing and determined by using the spectrophotometer at 412 nm against the blank.

2.4.5. Histopathological evaluation

The cross section full thickness skin sample was collected with the help of diethyl ether for anaesthetized the rats. On 18th day wound tissue sample from each group were collected. The specimen were fixed in 4% *p*-formaldehyde in PBS (pH 7.4) and then cut into 4 µm thick-sections and stained with hematoxylin and eosin to assess the predominant phase of wound healing [48].

2.4.6. Statistical analysis

Data are presented as the mean ± standard deviation. Treated groups were compared with the standard group. The results were analyzed statistically using Student's *t*-test for the comparison. The data were considered significant at $p < 0.01$.

3. RESULTS AND DISCUSSION

3.1. Isolation of Kaempferol

The previous study confirms that alcoholic leaves extract of *I. carnea* enclosed with flavonoids as well as kaempferol bioactive which have been claimed for antidiabetic action [16] antibacterial assets [12], and effective antioxidant [49]. The isolated composite was yellowish amorphous crystals, soluble in water, hot ethanol, methanol and ether with 274-275°C melting point. The outcomes of FTIR spectroscopic established the absorption band for Aromatic C-H stretching at 3215, 2887 cm^{-1}, and bending vibration approximately at 1379 cm^{-1}. In the spectrum of OH vibration, at approximately 3317, and others at 3172 cm^{-1} that are most probably of vibration of phenol OH group. The intensive band at 2613, 1660 (C=O linkage of a phenolic ring), from central heterocyclic ring, while the 1089, 1008 (C-O linkage in a heterocyclic aromatic ring), OH vibrations of the hydroxyl group occur at 1515, 1364, 1251, 1143 cm^{-1}, C-H vibrations of the unsaturated part appear at 785cm^{-1}. The 1H NMR spectrum exhibited a broad characteristic signal of proton signals at δ 12.5 (singlet 1H, OH-5, J = 0.096 Hz), δ 8.042 & δ 8.020 (doublet, J = 1.91 Hz, 2H,H-2,H-6), δ 6 .923 & 6.90 (doublet, J = 2.17 Hz, 3H, H-3, H-8), 6.487 (doublet, J = 0.932 Hz, 1H, H-6), 5.29 (doublet, J = 1.00 Hz, 1H, H-1). The 1H-NMR data were compatible with the previous literatures of kaempferol.

The molecular ion peak (m/z) 285.94 calculated against 286.24 m/z for MH^+ of kaempferol ($C_{15}H_{10}O_6$). Reports of comparative data of the spectral data with those of 3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one, compound was identified as kaempferol [50].

3.2. *In-vitro evaluation of dextran-PEG-chitosan-nano Titaniumdioxide-hydrogels* (DexCH-nTiO$_2$-HGs)

The topography, local surface modulus and shape of DexCH-nTiO_2-HGs imaging was conducted under AFM and HGs were found in µm size range having gel surface shown in (Fig. 1 A and B). The imaging of AFM demonstrates that the atomic force microscopic images are freshly prepared DexCH-nTiO_2-HGs. DexCH-nTiO_2-HGs were envisaged and establish to be relatively constant height, which screening the size of HGs with help of AFM. Contrasting other microscopic techniques, the AFM proposed revelation in three dimensions. The good behavior of HGs is prepared which confirm the AFM results.

The consequence of chitosan on the mechanical strength in gel structure was performed (Figure 2). With the adding the chitosan and dextran polymers, the machanical strength were decreased, whereas by deliberating the nTiO_2, it was improved. Many synthetic and natural polymer resources have been introduced for the treatment of simple and diabetic wounds, and restriction in their relevance since their weak mechanical assets and their poorer water absorption rate. In present work, with consideration to the outcome, established, that the mechanical properties will enhance by adding TiO_2 to the Dex-CH HGs. As observes in the elongation-force curve, which is the same to Stress-strain curve, in Figure 2), in a constant force strength, with the chitosan and dextran, the elongation of the gel structure is reduced since chitosan origins an enhance in pores in the membrane, thus lessening the crosslinking. In addition, found that by adding nanoTiO_2 escorts to enhance in elongation as TiO_2 enhances the tensile strength by blocking the pores presented in the gel structure.

The swelling property (water absorption ratio) was examined up to 6 hrs to evaluate their capability to retain wet atmosphere which is an essential aspect while it is applied onto an open wound surface. The effect of the DexCH-nTiO_2-HGs confirmed definite higher water absorption assets than the Dex, CH and nTiO_2-HGs. In all case, the swelling ratio of the hydrogels enhances with period of time. Within the 3 hrs, the DexCH-nTiO_2-HGs achieved to equilibrium state, while approximately after in 5 hrs the Dex, CH and nTiO_2-HGs absorption ratio accomplishes the equilibrium state of shown in Fig. 3.

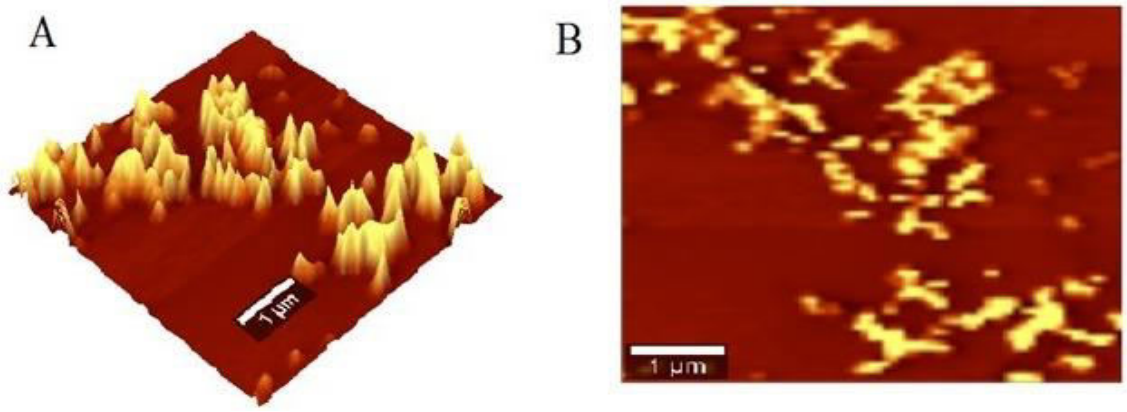


Fig. 1: AFM microphotographs of (A) 3D Hydrogel-network structure (B) pore size of DexCH Hydrogels

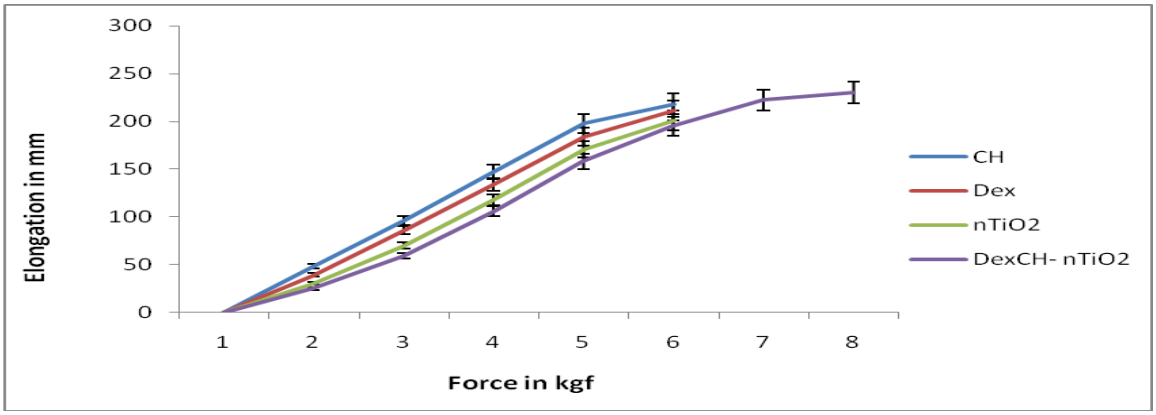


Fig. 2: Force-Elongation curve

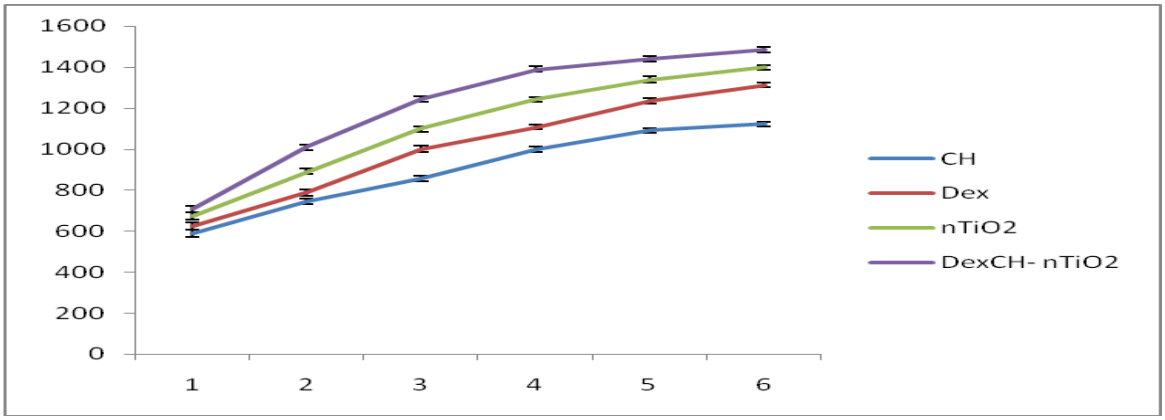


Fig. 3: Swelling behaviour of Dex, CH and DexCH- nTiO_2 **Hydrogels**

The swelling behavior and elastic modulus of the HGs is based on the theory of an arrangement of chains of cross-linking agents, amount of polymer after the design of gel. Enhancing number of ionic groups of polyelectrolytes in hydrogels is recognized to enhance HGs swelling behavior, and the itinerant responding ions in the gels prepration, a huge water absorption force due to some intermolecular non-covalent interface, that is polar forces and hydrogen bonding [51]. The mixing of nano titaniumdioxide ($nTiO_2$) doesn't much influence swelling property for hydrogels. Consequently, the augment of polymeric interactions originate an incredibly high swelling ratio. There is an opposite elasticity force, beside the favorable osmotic force which evade the deformation and balance the stretching of the HGs network [52].

The percent entrapment efficiency of optimized **Dex-Ch-** $nTiO_2$- **HGs** formulation was 89.36 ± 1.19%. 76.23±1.50% and 78.98±0.5 % were the entrapment efficiency of **Dex-HGs and Ch-HGs** formulations respectively shown in Table 1.

Table 1: Entrapment efficiency of optimized DexCH, GG and CH hydrogels

S. No.	Formulation	% Entrapment Efficiency
1	DexCH-$nTiO_2$-HGs	89.36 ± 1.19%
2	CH –HGs	76.23 ± 1.50%
3	Dex-HGs	78.98 ± 0.5%

Increasing with the amount of drug, were found to be increased in the strength of entrapment, resulting of impenetrable and high amount of drug entrapment. The reason behind that, enhance in drug concentration which increases crosslinking results, piloting to enhancing in drug holding potential. So the consideration can be made the greater degree of cross-linking by PEG as the quantity of Kaempferol increased. Accordingly, the probability of rigidity of developed insoluble complex matrices and cross-linking of the polymer were induced, ensuing in the DexCH-$nTiO_2$-HGs entrapped by extra drug**.**

Controlled and sustained applications provide numerous worthy assets against the currently available cutaneous drug delivery system for the care of dermal injury. The preferred steady amount of drug at the location of application, diminish the strength and decrease systemic drug level for toxic consequence recommend by the local controlled release system [53].

The elimination of delivery system from skin after the treatment not essential if biodegradable polymer and HGs is a competent source of wound remedial because of its chemical purity mechanical and physical assets. The HGs is the emerging trend to demonstrate quick preliminary release of drug (Kaempferol) which was pursued through steady release over longer time duration, showed in Fig. 4. A distinct time persistence release was establish of the KMF from the DexCH-$nTiO_2$ hydrogel. In 32 hours release percent of drug (Kaempferol) from DexCH-$nTiO_2$-HGs was found about 94.66%. Whereas percent release of CH-HGs and Dex-HGs and $nTiO_2$-HGs was found 83.51%, 79.51% and 76% respectively in 8 hrs which was shown in fig. 4.

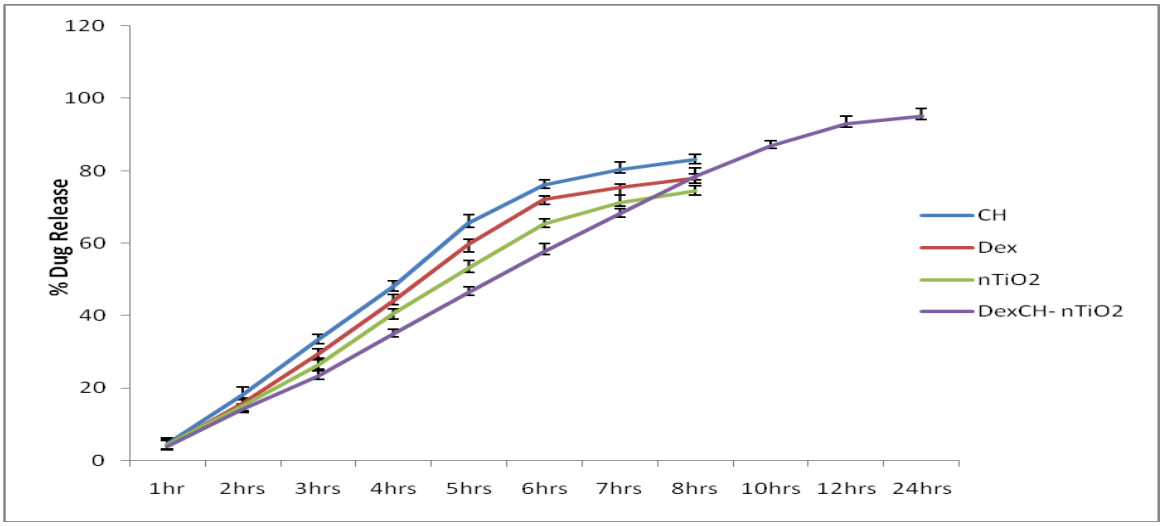


Fig. 4: *In-vitro* kaempferol release from CH, Dex, $nTiO_2$ and DexCH-$nTiO_2$ hydrogels

This is because of the collapsing the strong hydrogen bonding between Dextran-water molecules and Dextran-PEG-Chitosan-nano Titanium-dioxide polymers to liberate COOH groups for the crosslinking effect with the NH_2 group of bifunctional PEG (COOH-PEG-NH_2). The crosslinking dextran polymer chains develop into less soluble after the utilization of carboxyl group by PEG polymer. Dextran terminal have higher swelling in SWF (pH 7.5) which was considered for delayed release and water content of the swollen HGs is liable for rate of drug release [43]. The loading of drugs into the gel matrix, and consequent drug release on the particular time duration followed by the diffusion coefficient of the minute particles or macromolecule by the hydrogel network, which allow the great porosity arrangements of hydrogels [54].

3.3. *In-vivo* study of Dextran-PEG-chitosan-nano Titanium Dioxide- hydrogels (DexCH- nTiO$_2$-HGs)

Repair of wound is a dynamic complex phenomenon, incorporated sequence of biochemical, cellular and physiological process [55]. Formation of new blood vessels appears to be regulated by either vasculogenesis or angiogenesis arbitrated with endothelial cell interaction with the specific three-dimensional extracellular matrix atmosphere in the wound break. The new tissue growth by permitted adequate distribution of nutrients to sustain tissues metabolic needs, maintain by angiogenesis. It increasingly ensues with elongation and sprouting of new capillaries from the blood vessels of the intact tissues around the wound. Scars are also hypoxic, encompasses surplus of erratically shaped endothelial cells, and of inflammation that is an effective stimulator of angiogenesis [56]. Present research commences relevant topical preparation intended to proficient wound remedial.

The efficacy of novel natural products has been exploitation approach for the invention of pioneering medications. Previous research has designated that multiple inhibition of cell proliferation, migration, and biological effects possessed by flavonoids [57]. Commonly, HGs with is obliging as medical remedies in wound therapeutic and tissue production. The consequence of in-vitro study was bearded in concord among minute pore size HGs and high interconnection structure for *in-vivo* wound healing study [58].

3.4. Wound contraction measurement

The interval of each 2 days wound area was calculated by marking out on a transparent paper. Through subtracting from the original wound area, the cured region was measured. On day 6, the wound contraction of KMF fabricated DexCH-nTiO$_2$-HGs treated groups was found to be significantly ($p < 0.01$) improved which promoting to fast wound remedial as established by decreases epithelialization phase in comparison to control group. On day 18, KMF loaded DexCH-nTiO$_2$-HGs treated group was almost (100.64±1.32*) at absolute healing phase, while control group showed 85.42±2.12 % healing on day 20 (shown in Table 2 and Fig. 5). It was also experiential that epithelialization period of KMF loaded DexCH-nTiO$_2$-HGs and standard groups were less in comparison to control group (Table 2).

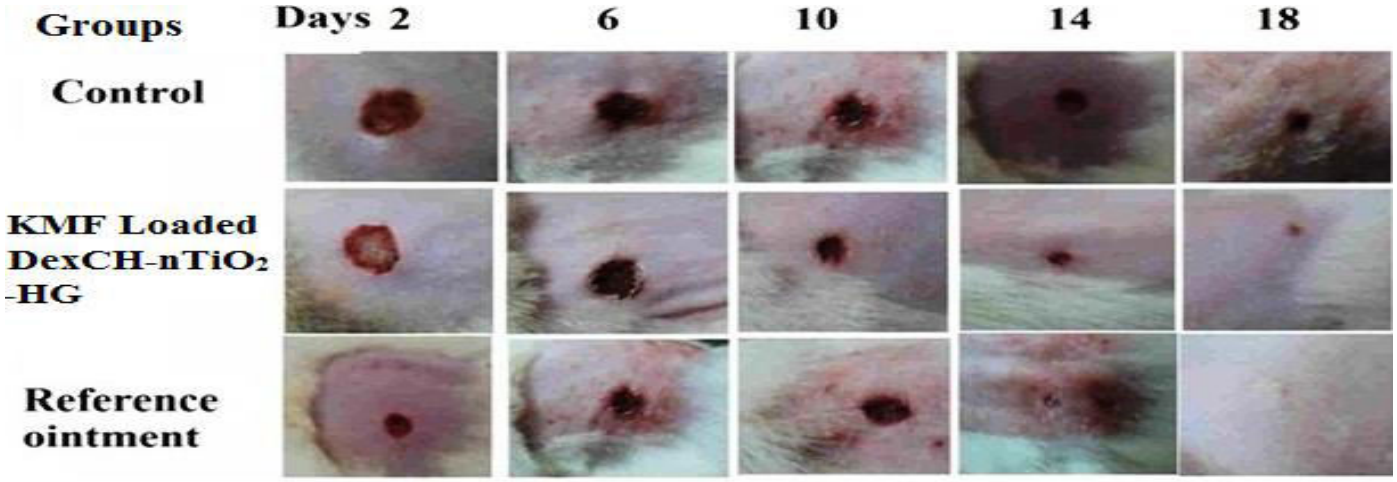
Table 2: Effect of prepared Kaempferol loaded DexCH-nTiO$_2$-HGs and reference ointment on percent wound contraction area of diabetic wound in rats

Groups	Post wounding days (Percent wound contraction)										Epithelia-lization period
	2	4	6	8	10	12	14	16	18	20	
Control	14.53±1.60	28.37±1.12	36.38±2.08	39.63±2.21	46.47±2.21	53.11±2.23	61.41±1.31	70.20±1.83	79.62±1.05	85.42±2.12	23
Kaempferol loaded DexCH-nTiO$_2$-HGs	15.38±1.58	26.28±2.06	43.48±2.04	49.84±1.84	60.49±1.87	70.40±2.32	83.64±1.83*	93.71±2.10*	100.64±1.32*	-	18
Reference Ointment	16.78±2.24	29.36±2.14	45.81±2.03	50.25±1.79	65.62±1.93	73.54±1.84	86.61±2.31*	94.05±2.02*	100.17±1.24*	-	18

*n = 6 albino rats per group, value represents Mean ± S. *p < 0.01, when compared each treated group with control group*

3.5. Tensile strength study

The tensile strength of the wounds treated KMF loaded DexCH-nTiO$_2$-HGs (366.5±17.74) and reference ointment (370.5±31.69) was found significantly (P< 0.01) higher than that negative control (193.4±16.41) and Control (207.6±18.25) of animal respectively. Slightly increase tensile strength was found KMF loaded DexCH-nTiO$_2$-HGs (366.5±17.74) (Table 3). The enhance wound healing action is well observed by the determination of tensile strength of the incised wound. The molecule with wound remedial activity commonly offer enrichment in the collagen content deposition, which supply potency to the tissues and outline the cross-linkages among collagen fibers [59].



Control (without Kaempferol DexCH-nTiO$_2$-HGs), Kaempferol loaded DexCH-nTiO$_2$-HGs and reference group (marketed formulation 'Povilet' Ointment) in diabetic wound model

Fig. 5: Wound areas of different groups

Table 3: Effect of prepared formulations on tensile strength of tissue from incision wound model in rats

Groups	Tensile strength (gm/cm^2)
Control	207.6±18.25
DexCH-nTiO$_2$-HGs	366.5±17.74*
Reference Ointment	370.5±31.69*

*n= 6 albino rats per group, value represents mean ± S.D. *P< 0.01, when compared each treated group with control group*

3.6. Collagen content, protein level and granuloma weight quantity

The increased weight of the granulation tissue also indicated the presence of higher protein content for KMF loaded DexCH-nTiO$_2$-HGs was 72.58±1.25 and reference ointment group was 73.08±1.73, animals shows considerably enhance than control group (48.04±2.54). The consequence of topical management of the KMF loaded DexCH-nTiO$_2$-HGs treated group on dead space wound model was assessed by increase 30.67±1.23 in the hydroxyproline level when compared to control group 19.21±2.54 and the reference group was shows 31.32±1.14 (Tables 4). The granuloma weight of dead space wound for KMF loaded DexCH-nTiO$_2$-HGs (37.32±2.17 mg) and reference ointment treated (36.75±2.32mg) group of animals shows significant ($p<0.01$) higher against the control group. The protein synthesis encouraged by development of granuloma tissue. The cellular proliferation as well as levels of protein synthesis confirms the development of the protein content of granulation tissues. Increase in protein contents of the treated wounds compared to control group recommend that KMF, encouraged production of new cells through an unidentified system. The infiltration of macrophages, fibroblasts and neutrophils production are required for the Inflammation stage which is the fundamental resource for granuloma tissue development [60]. Hence the development of the proliferative phase in addition to the existence of higher protein level specifies enhance in granuloma weight.

In the extracellular matrix collagen is available as chief element and contributes to the tensile strength of wound. Hydroxyproline and its peptides are arising during post-translational modification of enzyme and collagen [61]. The synthesized new collagen candidates are covered at the wound location and taking place cross linking to construct fibers. The potency of wound is gained through the development of intra and inter-molecular cross links and refurbishment of collagen. Previously confirmed that the use of flavonoids kaempferol clearly reveals collagen density and increased dermal thickness compared with dimethyl sulfoxide culture [62]. Consequently the increased collagen turnover and escort to rapid healing of the treated wounds was specified increased in hydro-xyproline level of the KMF loaded DexCH-nTiO$_2$-HGs treated wounds.

3.7. Estimation of antioxidants level in skin tissue

The KMF loaded DexCH-nTiO$_2$-HGs have effective antioxidant action by augment in the SOD (19.32±1.87

μg/50 mg tissue), GSH (23.52 ±2.51μmol/50 mg tissue) and catalase level (29.56±2.27μmol/50 mg tissue) in the granuloma tissues for wound therapeutic course (Table 5). On 9th day in diabetic wounds, the considerable progress in SOD, GSH and CAT level in KMF loaded DexCH-nTiO_2-HGs and reference ointment groups be established.

The strong free radical scavenger is reduced glutathione which protects the membrane lipid from the oxidant aggressions by contributing the protons to membrane lipids. The GSH is other defense mechanisms against free radicals like the enzymes superoxide dismutase (SOD) and catalase (CAT). These activities contribute to eradicate superoxide, hydrogen peroxide and hydroxyl radicals along with the reduction of GSH consequences in improved lipid peroxidation. These are main basis to enhanced GSH operation and can be associated to the increase in the level of oxidized glutathione [63]. Application with of KMF loaded DexCH-nTiO_2-HGs showed enhance the GSH levels, which control the redox status of protein in the membrane against oxidative injury and defend the cell membrane. The enzyme SOD and CAT offer the antioxidant resistance that obliterates peroxide to an organism. The improved oxidative stress and deposition of lipid peroxides take place in injured area, if the poorer enzymes behavior. During the chronic wound, the performance, of these enzymes is increased when apply the management of KMF formulation and hence could assist to rise above from free radicals fabrications.

Table 4: Effect of Kaempferol loaded DexCH-HGs and reference ointment on different biochemical parameters of Dead space wound in rats

Groups	Hydroxyproline (mg/ g tissue)	Protein content (mg/g tissue)	Granuloma weight (mg)
Control	19.21±2.54	48.04±2.54	18.10±2.88
Kaempferol loaded DexCH-nTiO_2-HGs	30.67±1.23*	72.58±1.25*	37.32±2.17*
Reference Ointment	31.32±1.14*	73.08±1.73*	36.75±2.32*

*n = 6 albino rats per group, value represents Mean ± S.D. *p < 0.01, when compared each treated group with control group*

Table 5: Effect of Kaempferol loaded DexCH-HGs and reference ointment on different biochemical parameters of diabetic wound in rats

Groups	SOD (μg/50 mg tissue)	CAT (μmol/50 mg tissue)	GSH (μmol/50 mg tissue)
Control	10.38±2.34	17.74±1.78	11.31±3.31
Kaempferol loaded DexCH-nTiO_2-HGs	19.32±1.87*	29.56±2.27*	23.52 ±2.51*
Reference Ointment	19.56±1.42*	28.34±2.53*	22.42±2.27*

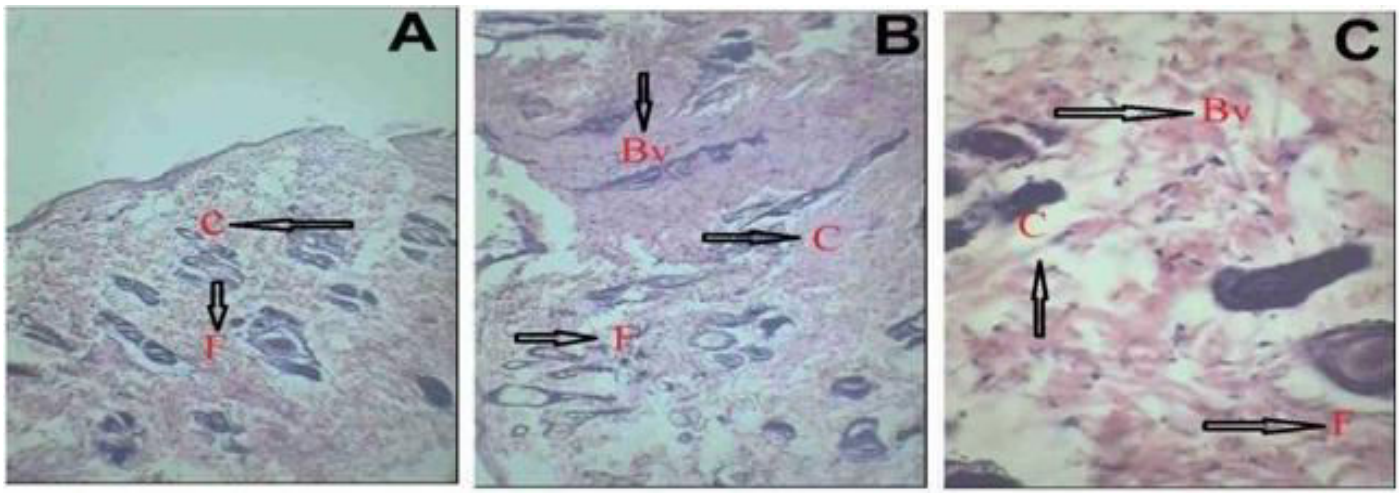
*n = 6 albino rats per group, value represents Mean ± S.D, *p < 0.01, when compared each treated group with control group*

3.8. Histopathological study

Histopathological study of stained section from different treated groups was examined for cellular epithelial regeneration and matrix organization and reveal diverse healing circumstances of the injured tissues. It validates by good evidence of the hydrogel formulation outcomes of suitability for promoting healing in normal and diabetic wounds (Fig. 6). The study illustrated that the original regeneration of tissue found efficiently in the wound treated with KMF loaded DexCH-nTiO_2-HGs and reference group with no any congestion and edema. The granulation tissue section of control animals illustrated diminished epithelialization, fibrosis and more aggregation of macrophages with less collagen fibers, indicating imperfect healing of wounds. The proliferation of epithelial tissue covering the injured area showed in both group. The dermal modeling process was very slow in control group which was showed by the lower epithelialization period. Interestingly the animals treated with formulation showed fibrous connective tissue with well-collagenation and complete healing. An increase in blood vessels, fibroblast cells growth and collagen fibers confirms in histopathological examination. Rahman, 2007 stated that natural antioxidants are biological molecules readily absorb and be active directly or indirectly as antioxidants by chelate redox metal at physiological stage and quenching a free radical scavenging [64].

In this research, from ethanol extract of *I. carnea* leaves, isolated the pure kaempferol (KMF) and studied for relevance for wound healing of prepared KMF- DexCH-

$nTiO_2$-HGs. The consequences showed reduced healing time and enhanced rate of wound contraction in treated with KMF HGs animals [65]. These results are reliable with other information of wound healing activity of *Ipomea carnea*. The naturally occurring flavonoids, of *I. carnea* extract, have been reported for screening of antioxidant activity on different model in rats [66, 67]. Flavonoids are used for several remedial assets for example antioxidant, anti-inflammatory, wound healing with antifungal activity as well as identified to persuade the fast wound healing due to antimicrobial property [5, 8, 10]. Thus, prospective of wound healing for *I. carnea* might be recognized chemical components in the leaves, due to their interdependent effect that increased the proliferation stage of wound healing.



Hematoxylin and eosin,×100; (A) Control (without Kaempferol DexCH-nTiO$_2$-HGs); (B) Kaempferol loaded DexCH-nTiO$_2$-HGs (C) Reference group (marketed formulation 'Povilet' Ointment); F: Fibroblast cells; C: Collagen fibers, Bv: Blood vessels

Fig. 6: Photomicrograph of rat skin tissues after post-wounding days for different treatment groups in diabetic wound model

4. CONCLUSION

The isolated kaempferol from ethanolic fraction of *Ipomea carnea* Jacq. promotes the potent wound healing and antioxidant activities for normal and diabetic injuries. It may be due to the ability to scavenge free radicals, an increased cross-linking and in dry granuloma weight which specified elevated protein content in tested animals. The ethnopharmacological approach of chooses flavonoid kaempferol study may be valuable for transdermal or controll drug delivery applications. There is not much information available on the phytochemical and pharmacological studies on kaempferol for wound healing potential. The report of the efficacy of this, constituent (Kaempferol) as wound healing may be due to the prepared Hydrogels (KMF-DexCH-nTiO$_2$-HGs) have improved drug entrapment, good swelling property and sustained release capability which is dynamic aspects for decreasing the hazard of wound dehydration . The prepared hydrogel with PEG as crosslinker showed the excellent result against the previous reported dextran chitosan based hydrogel. In conclusion the result of present study offers the pharmacological evidence for the use of kaempferol for potent wound healer as hydrogel formulation. The Kaempferol loaded DexCH-nTiO$_2$-HGs biodegradable polymer based hydrogels, accelerate the wound closure and promoting extracellular matrix remodeling of wounded tissue by acting in the remodeling phase of the wound healing course and rationalize the folklore remedies.

5. ACKNOWLEDGEMENT

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