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Bromelain-loaded chitosan nanofibers prepared by electrospinning method for burn wound healing in animal models

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ABSTRACT

Bromelain is a mixture of proteolytic enzymes present in all tissues of pineapple (*Ananas comosus*). It is known as an efficient debriding agent in burn treatment. In this study, the efficiency of bromelain-loaded chitosan nanofibers for burn wounds repair was investigated in animal model. Chitosan nanofibers containing bromelain (2% and 4% w/v) were prepared by electrospinning method. The physicochemical characteristics of the synthetized nanofibers were evaluated. The release profile and activity of bromelain loaded in nanofibers were also assayed. Cytotoxicity test was carried out using Alamar blue. The burn healing effect of chitosan-2% w/v bromelain nanofiber was studied in the induced burn wounds in rats for 21 days. The efficacy of treatment was assessed by reduction of burn wound area and histological characteristics at different times. Chitosan-2% w/v bromelain showed the better physicochemical properties and release profile as well as low cytotoxicity than chitosan-4% w/v bromelain. The results also indicated that chitosan-2% w/v bromelain nanofiber was more efficient to heal burn skin compared to chitosan nanofiber alone in the animal model tested. The present study concludes that chitosan-2% w/v bromelain nanofiber possesses great wound healing activity and could be considered as an effective natural topical burn wound healing treatment.

1. Introduction

Burn injury is still a major public health problem resulting in the increase of the preventable deaths and disability rate every year. The aim of burn treatment is enhancing the burn healing process including proliferation, granulation, epithelialization, and collagenation [1,2]. There are different synthetic and herbal medicines to facilitate the process of wound healing. However, herbal products have attracted increasing attention in treatment of burn wounds due to cost-effectiveness and low adverse effects [3,4]. Bromelain is a crude extract from pineapple which contains proteolytic enzymes [5]. The therapeutic effects of bromelain have been demonstrated in different diseases such as angina pectoris, bronchitis, sinusitis, surgical trauma, thrombophlebitis, osteoarthritis, diarrhea, cancer, and various cardiovascular disorders. It has been also used for treatment of wounds, burns, and inflammations [6–12].

Different nanoformulations based on silica, gold, poly(acrylic acid), poly lactic-*co*-glycolic acid (PLGA), katira gum, chitosan, niosome and lipid core nanocapsule have been developed for delivering bromelain in order to enhance its activity, stability and decrease its toxicity [7,13,14]. Electrospinning has been considered as an efficient technique to produce nanometer scale polymeric fibers. The main advantages of electrospinning method include: controlling the nanofiber composition to achieve the desired property, decreasing the adverse effects of systemic medications, and creating effective concentration of drug in the damaged area. In addition, due to high surface-to-volume ratio, the loading of enzyme and other biological agents can be easily accomplished [15,16]. Chitosan, a linear 1, 4-linked polysaccharide, is biodegradable, nontoxic, and antimicrobial polymer. Previous studies showed that this polymer has good biocompatibility and positive effects

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on wound healing [17,18].

In this study, according to optimal properties of surface to volume of the electrospun nanofibers and appropriate properties of chitosan and bromelain enzyme in burns treatment, the chitosan-bromelain (Ch-Br)-nanofibers were produced by electrospinning device. The physico-chemical properties of nanofibers, drug release profile, enzyme activity, cytotoxicity, and finally the potential of synthetized nanofibers in the recovery of burned skin in animal model were evaluated.

2. Experimental methods

2.1. Material

Low molecular weight chitosan was obtained from Sigma Aldrich (Germany). Bromelain powder was purchased from Sigma (USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco (USA). Animals were purchased from Institute of Medical Science, Mashhad, Iran. Gelatin was prepared from Molekula (UK). BCA kit was bought from Parstoos Company (Iran). Trichromason, hematoxylin and eosin stains were purchased from Merk (Germany). To anesthetize the Rats, xylazine 2% was purchased from Alfasan Company (Netherland) and ketamine 50 mg/ml from Trittau (Germany).

2.2. Preparation of electrospinning solution

Chitosan solution was prepared by dissolving 0.625 g of chitosan in 25 ml acetic acid (90% v/v) following by stirring for 24 h at room temperature. To obtain chitosan-2% w/v and 4% w/v bromelain (Ch-Br) nanofibers, 200 and 400 mg of bromelain was dissolved in 1 ml of deionized water and then mixed with 8 ml of chitosan solution and 1 ml of polyethylene oxide 2.5% w/v and stirred for 10 min to get the homogenous solution.

2.3. Viscosity and electrical conductivity measurement

The viscosity of all solutions was measured using a Brookfield R/S + Rheometer (Brookfield Co., USA) rotational rheometer with CC25 spindle at a shear rate of 0–200 s^{-1}. Electrical properties were also evaluated by Conductivity Meter 8302A (Goldpoint Co. Ltd., Taiwan).

2.4. Electrospinning process

The solution was loaded into syringe equipped with the stainless steel needle of 0.9 mm in diameter (gauge 18). The filled syringe was placed into the electrospinning device (Fnm Co., Iran). The applied voltage of 10 kV, needle to the collector distance of 200 mm, and flow rate of 0.5 ml/h were applied for electrospinning process. The electrospun fibers were collected on the rotating drum covered with aluminium foil (200 rpm) at 25 °C.

2.5. Crosslinking process

In order to increase the stability of nanofibers in aqueous medium, crosslinking was performed using glutaraldehyde vapor [19]. Briefly, the fibers were placed in a sealed desiccator containing 10 ml 25% (v/v) glutaraldehyde aqueous solution for 5 h. After cross linking, nanofibers were moved to the vacuum desiccator for 24 h.

2.6. Characterization of synthetized nanofibers

Microscopic observation of the membranes was performed using a Field Emission Scanning Electron Microscope (FE-SEM) (MIRA3, TESCAN CO., Czech). Fourier transform infrared spectroscopy (FTIR) study was carried out at wavelength range of 4000–450 cm^{-1} (Spectrum Two, PerkinElmer, USA). The thermal behavior of nanofibers was characterized by differential scanning calorimetry (DSC) in the temperature range 20–350 °C with a heating rate of 10 °C/min (DSC10, Nanjing Dazhan Institute of Electromechanical Technology, China). The mechanical properties of the nanofibers were examined using a universal testing machine (H50KS, Hounsfield, UK). The specimens were cut into strips of 1 × 4 cm. After measuring the thickness by a digital micrometer (293 MDC-MX Lite, Mitutoyo, USA), the samples were mounted into the grips and stretched with a strain rate of 5 mm/min until breakage. Five independent measurements were done and the means ± standard deviations (SD) of young's modulus were reported.

2.7. Swelling test

The swelling ratio was measured by immersing the pre-weighed dry cross-linked nanofiber in phosphate buffer saline (PBS) with pH 7.4 [20]. After 4 and 24 h, the percentage of the swelling samples was calculated according to the following formula

$$\mathrm{WC}(\%) = \frac{(W - W_0)}{W_0} \times 100$$

where WC is water capacity, W_0 is the initial weight of dry sample, and W is the weight of swollen material after determined time.

2.8. Loading analysis

Loading efficiency was calculated by weighing 2 mg of Ch- 2% and 4% w/v Br nanofibers and dissolving them in 1 ml acetic acid 2% v/v. Then the amount of bromelain was measured by the BCA Kit. Loading efficiency of bromelain was computed by the following equation:

$$\text{Loading efficiency}\ (\%) = \frac{\text{amount of Bromelain loading in nanofiber}}{\text{total amount of used Bromelain}} \times 100$$

2.9. In vitro release study

In vitro release profile of bromelain from Ch-2% and 4% w/v Br were carried out in PBS [21]. Briefly, 2 mg of each cross-linked nanofibers were cut and suspended in 1 ml PBS. The samples were kept in shaker incubator at 37 °C with constant stirring. At determined time interval (1, 2, 3, 4, 24, 48, 72, 96, 120, 144, 168, 192 h), the nanofiber membranes were moved into fresh PBS. The amount of bromelain in solutions was examined using BCA Kit.

2.10. Enzymatic activity of bromelain

The enzymatic activity of bromelain was assayed as described previously [22]. Briefly, 2 mg of each cross-linked nanofiber was immersed in 1 ml of PBS 0.1 M, pH 7.4. After 1 and 4 h, 100 µl of released sample was mixed with 2.5 ml of gelatin 5% w/v, heated up to 80 °C and then cooled to 45 °C. Bromelain as a proteolytic enzyme, degrades gelatin into amino acids and oligo peptides. Gelatin alone was considered as the blank (containing no enzyme). The bromelain at concentrations of 1, 2, and 4% w/v in PBS were also used to compare the bromelain activity before and after formulation. All samples were left at the room temperature for 20 min. Then, 10 µl of H_2O_2 3% v/v was added to each solution to stop the reaction followed by adjusting the pH to 6.9 with NaOH 0.05 M. After that, 1 ml of formaldehyde 37% v/v was added to each sample and pH was again adjusted to 7.8 using NaOH. The volume of needed NaOH (mL) represented the amount of enzyme activity of bromelain.

2.11. Stability of enzyme activity

In order to determine time depended enzyme activity of bromelain, the cross-linked Ch-2% w/v Br nanofiber was stored at 4 °C for

6 months [23]. Then, 2 mg of nanofibrous membrane was cut and floated to 1 ml PBS and located in shaker incubator for 1 and 4 h at the 37 °C. Then, the enzyme activity was measured as described in Section 2.10.

2.12. Cytotoxicity test

Cytotoxicity of synthetized nanofibers was determined using Alamar blue assay [20]. The punched membranes were placed into 96 well plate and sterilized with plasma sterilizers (Renosem, Bucheon-si Co, Korea). The human dermal fibroblasts obtained from skin samples were seeded on the membranes in 96 well plates containing DMEM, 10% FBS and 1% penicillin-streptomycin at density of 5×10^3 cells/well and incubated in a humidified incubator at 37 °C with 5% CO2. After 72 h, 10 µl of Alamar blue (10% v/v) was added to each well and the cells were further incubated for 3 h. Then, the Alamar blue absorbance was measured at an excitation wavelength of 570 nm and an emission wavelength of 600 nm using ELISA reader (Infinite M200, Austria). The percentage of cell growth was calculated using the following formula:

$$\text{cell viability (\%)} = \frac{\text{absorption of test sample}}{\text{absorption of the control}} \times 100$$

2.13. In vivo studies

For animal studies, the second degree burn was induced on the rat's skin (weight range 250–300 g). At first, the back of each rat was shaved well and then the animal was anesthetized with xylazine/ ketamine at the ratio of 1:1 through the biliary injection. The metal coin with the approximate diameter of 2.5 cm immersed in boiling water (100 °C) for 3 min and placed on the back of rats for 5 s. Sodium chloride 0.9% w/v (0.5 ml) was also injected to avoid hypovolemic shock [24].

The rats were divided into control and treated groups. Treated groups were dressed up with chitosan or Ch-2% w/v Br nanofibers (containing 1.5 mg bromelain) at different times (0, 1, 7, 14, and 21 days). Every two days, the dressing materials were changed till the end of study.

The biopsies of wound regions were obtained in each time points using 0.6 mm biopsy punch and fixed in neutral buffered formalin. The samples were then stained with both hematoxylin and eosin (H&E) and trichromason for pathological examination. Biopsy specimens were evaluated microscopically to study the burn degrees and some other parameters such as necrosis, infiltration of inflammatory cells, angiogenesis, and proliferation of fibroblasts, collagen deposition, and re-epithelialization.

2.14. Statistical analysis

The statistical software GraphPad Prism 7 was used in order to analyze the data. The comparison between different groups was done by *t*-test and one-way ANOVA. To compare the mean of data in different groups, the post test of Tukey-Kramer was used. In this study the $p < 0.05$ was considered as significant level.

3. Results

3.1. Viscosity and electrical conductivity

As shown in Table 1, adding bromelain into chitosan solution at concentration 4% w/v increased viscosity and electrical conductivity ($p < 0.0001$). However, there were no significant differences between the viscosity of chitosan and Ch-2% w/v Br solution ($p > 0.05$).

The results of electrical conductivity measurement showed that the highest electrical conductivity belongs to Ch-4% w/v Br solution (939 ± 20.2 µS/cm). No significant difference was observed between the electrical conductivity of chitosan 2.5% and Ch-2% w/v Br solution.

Table 1
The average viscosity of solutions in shear rate 100 S^{-1} and electrical conductivity.

Sample	Viscosity (mPa·S) mean	Electrical conductivity (µS/cm)
Chitosan 2.5%	100 ± 0.6	693 ± 19.3
Ch-2% w/v Br	110 ± 0.3	724 ± 17.5
Ch-4% w/v Br	170 ± 0.2	939 ± 20.2

3.2. Characterization of nanofibers

FE-SEM images showed that the nanofibers had smooth surface, however some beads were observed in the Ch-4% w/v Br (Fig. 1, A–C). Microscopic images depicted the increment in the average fiber diameter of nanofiber containing bromelain compared to chitosan nanofiber (Fig. 1-D).

The results of FTIR analysis were shown in Fig. 2(A–D). In chitosan spectrum, the broad peak at 3427 cm^{-1} corresponded to N—H and O—H stretching as well as intramolecular hydrogen bond. The peak around the 2877 and 2920 cm^{-1} confirmed the existence of C—H symmetric and asymmetric stretching, respectively. The peak around 1655 cm^{-1} and 1379 cm^{-1} is related to C═O stretching of the first amide and C—N stretching of the third amide, respectively.

On the other hand, the bonds around 3385 cm^{-1} and 1655 cm^{-1} corresponded to N—H stretching and carbonyl groups of bromelain, respectively. Loading bromelain into chitosan nanofibers resulted in changes in the peak of bromelain carbonyl group. The peak got wider and shifted from 1655 to 1622 and1627 cm^{-1} in Ch-2% and Ch-4% w/v Br, respectively. Furthermore, the peak at 3385 cm^{-1} in bromelain spectrum was also moved to 3269 and 3277 cm^{-1}. These changes could be due to the formation of intermolecular bond between chitosan and bromelain.

The loading of bromelain into chitosan nanofibers may alter the thermal properties, which was investigated by DSC. As seen in Fig. 2(E–H), the chitosan DSC curve exhibited endothermic peaks at 74.60 °C, which was due to water evaporation and the melting of PEO. The peak at 313.05 °C was also belonged to the destroying of chitosan polymer. In the DSC graph of bromelain, the peak at 80.66 °C was belonged to water evaporation and the peak at 250.46 °C was related to melting of Bromelain. Another peak at 325 °C was also observed that was corresponded to the destroying of drug. According to these results, it can be concluded that, by loading bromelain in nanofibers, the amount of all peaks in bromelain curve decreased and shifted to the left and with increasing the bromelain concentration in nanofiber, chitosan's peak decreased more. These alterations in peaks indicated the presence of bromelain in nanofiber and its effects on thermal characterization of the nanofiber.

3.3. Tensile properties

The tensile properties of synthetized nanofibers were evaluated. According to the results, the Young's modulus of non-crosslinking Ch-Br nanofibers in both concentrations (Ch-2% w/v Br with 220 ± 0.10 MPa and Ch-4% w/v Br with 210 ± 0.12 MPa) was significantly higher than chitosan nanofibers (164 ± 0.11 MPa) ($p < 0.0001$). The crosslinking of Ch-4% w/v Br nanofiber resulted in significant reduction in tensile strength. The Young's modulus of crosslinking chitosan and Ch-2% w/v Br nanofibers was 208 ± 0.13 (MPa) and 157 ± 0.09 (MPa), respectively.

3.4. Swelling test

The results of swelling test are presented in Table 2. The maximum swelling ratio was for chitosan 2.5%, then Ch-2% w/v Br and then Ch-4% w/v Br, respectively.

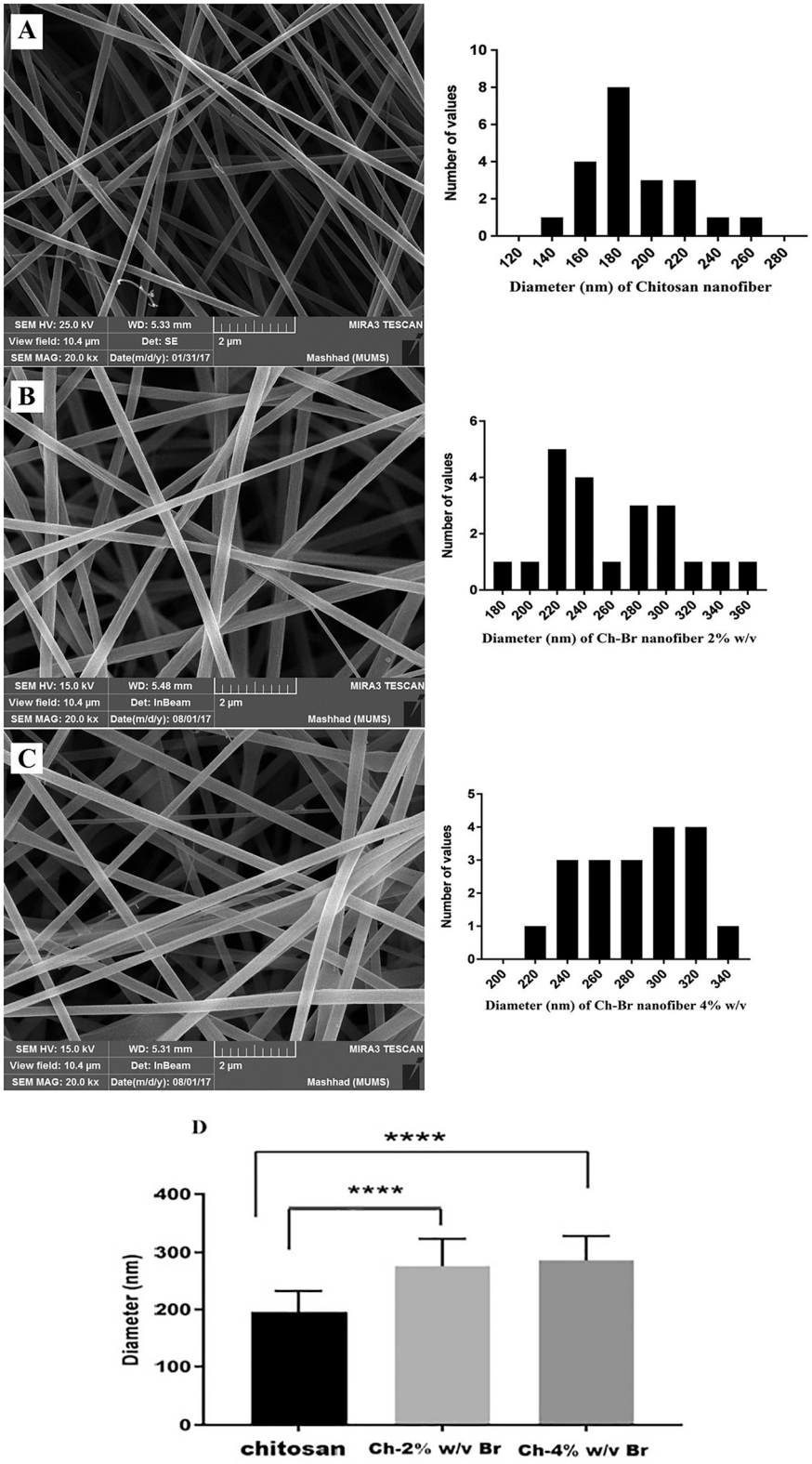

Fig. 1. FE-SEM images at 20,000-fold magnification and fiber diameter distribution of, A) Chitosan, B) chitosan-2% w/v bromelain and C) chitosan-4% w/v bromelain. D) Mean diameter of nanofibers in chitosan alone, Ch-2% w/v Br, and Ch-4% w/v Br. **** means $p \leq 0.0001$.

3.5. Loading analysis

Loading efficiency of bromelain in nanofibers was evaluated by BCA kit. It was observed that 91% and 96% of bromelain were loaded into Ch-2% and 4% w/v Br formulation, respectively.

3.6. In vitro release profile

The *in vitro* release profile of bromelain from Ch-2% and 4% w/v Br was evaluated in PBS (pH 7.4) for 8 days (Fig. 3). The results showed that Ch-2% w/v Br released the highest amount of Br in the first 24 h

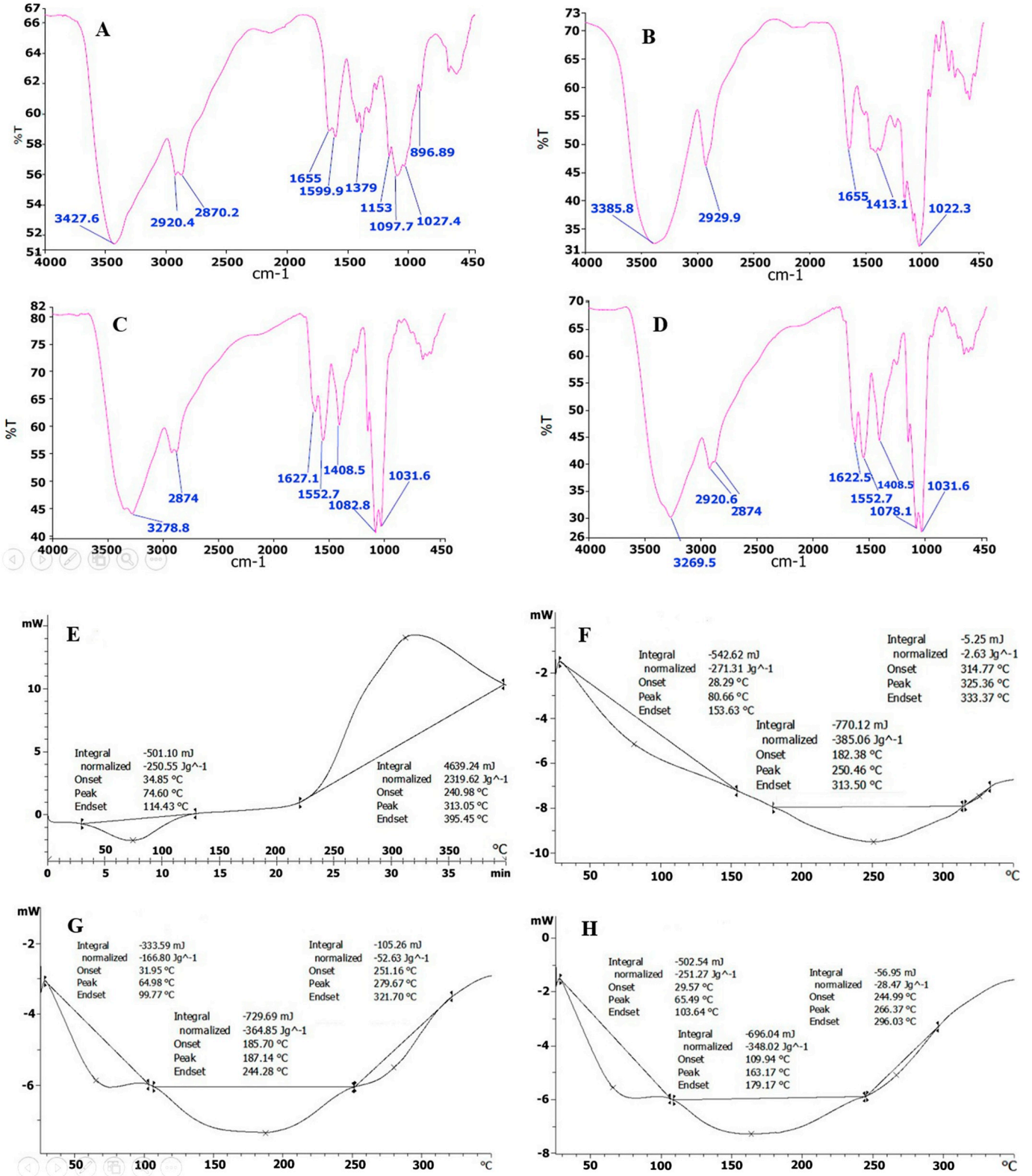

Fig. 2. The FT-IR spectra analysis. A) Chitosan nanofibers, B) Bromelain powder, C) Ch-2% w/v Br and D) Ch-4% w/v Br nanofiber. DSC Analysis. Chart. E) Chitosan nanofiber; F) Bromelain powder; G) Ch-2% w/v Br, and H) Ch-4% w/v Br nanofiber.

Table 2
The percentages of water capacity (WC %) of nanofibers.

Sample	After 4 h	After 24 h
Chitosan 2.5%	700% ± 32	1025% ± 36
Ch-2% w/v Br	635% ± 6	860% ± 6
Ch-4% w/v Br	357% ± 7.5	507% ± 7.6

(93%) but this amount had been released during 144 h for Ch-4% w/v Br and a slower release was observed in Ch-4% w/v Br formulation.

3.7. Activity of bromelain

The enzymatic activity of bromelain loaded in chitosan nanofibers is related to the volume of NaOH 0.05 M needed to neutralize H^+ ions. The results showed that 97.7 ± 1.98% and 96 ± 2.08% of bromelain activity was maintained in Ch-2% Br and Ch-4% Br, respectively.

3.8. Stability of activity

This examination proved that bromelain kept its activity in Ch-2% w/v Br nanofiber after 6 months storage at 4 °C. The results showed that, the nanomaterial had still 96 ± 3.12% of the activity of bromelain after 6 month storage.

3.9. Cytotoxicity test

The impact of Ch-2% and 4% w/v Br on cell viability was assessed using Alamar blue test. As shown in Fig. 4, no cytotoxicity was observed in chitosan, Ch-2% w/v Br, and bromelain with equal concentration in nanofibers. However, cross-linked Ch-4% w/v Br showed significant cytotoxicity in comparison with the control group ($p < 0.01$).

3.10. In vivo study

In Fig. 5-A, burn appearance at 1, 7, 14, and 21 days are shown in different groups. In twenty-one days, the burn area in the Ch-2% w/v Br group significantly decreased and was cured with almost complete skin and hair regeneration. In histological analysis using H&E staining, on day 1, epidermal and dermal damage with infiltration of inflammatory cells were observed in all three groups. On day 7, formation of granulation tissues including proliferation of fibroblast cells and angiogenesis was detected in all groups. Over the time, in the studied group, the density of fibroblast cells reduced and the density of collagen fibers increased. On the day 14, in the Ch-2% w/v Br group, the density of granulation tissue reduced, while they seemed to be more mature compared to other groups. In this group, the hyperplastic epithelium was also recognizable. On the day 21, Ch-2% w/v Br group had more regular collagen fibers than other two groups and granulation tissue was seemed to be more adult. In this group, the epithelium tissue was covered the entire surface of the wound while in other two groups,

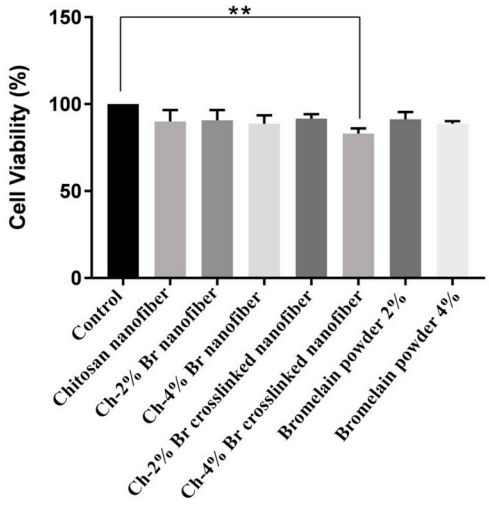

Fig. 4. Cytotoxicity analysis using Alamar blue test. Each analysis was done 3 times and data are shown as p value ≤ 0.01 with **.

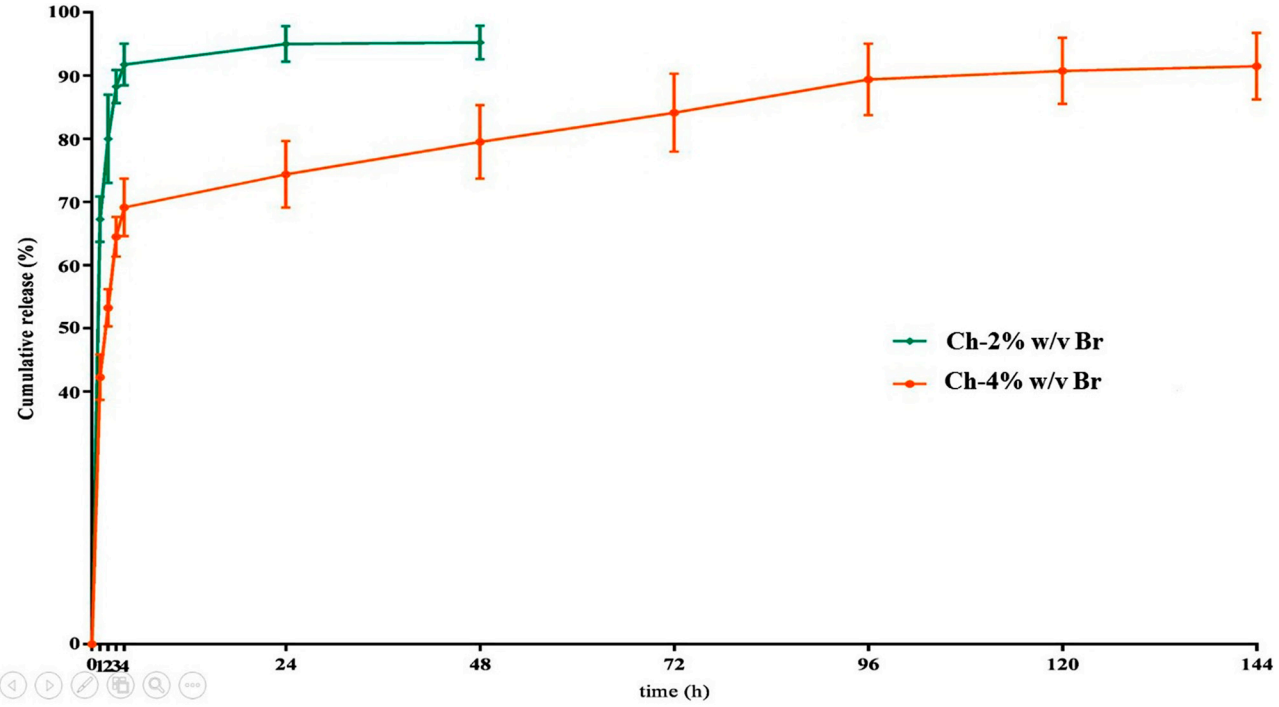

Fig. 3. Release of bromelain from Ch-2% w/v Br (green line) and Ch-4% w/v Br (orange line) in PBS (pH 7.4). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

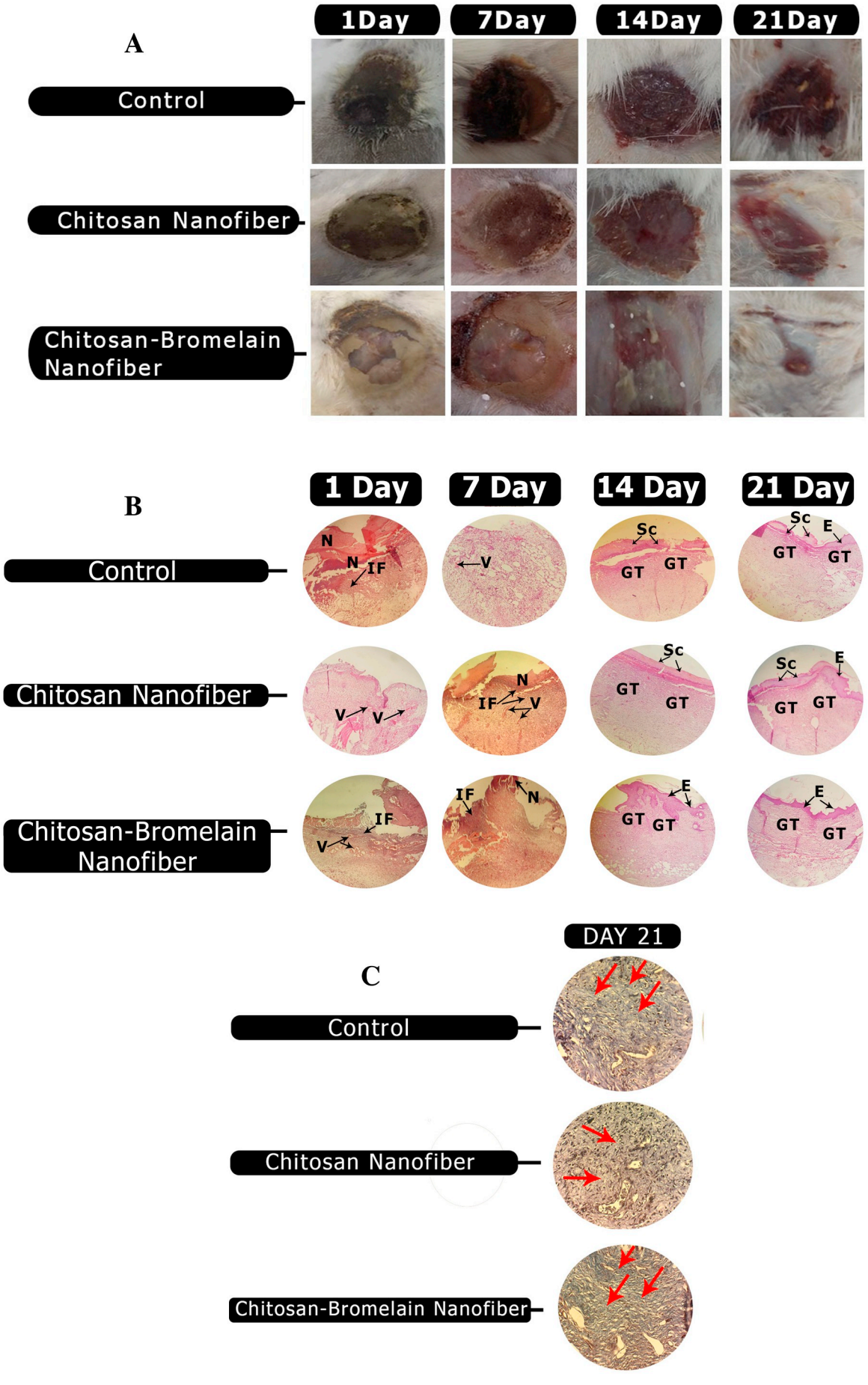

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Fig. 5. A) The vision of damaged tissues (2nd burn degree) which were created by heated metal coin with approximate diameter of 2.5 cm on rat's skin and the regeneration of skin. B) H&E studding on days 1, 7, 14, and 21 day, IF: Inflammatory cells, V: vessels, Sc: Scab, E: Hyperplastic Epithelium, GT: Granulation Tissue, C) Trichromason staining on day 21.

Table 3
Evaluation of measurable factors in burn healing. +: Slight, + +: Moderate, + + +: Marked, + + + +: Extensive, −: Absence.

Day	Group	Necrosis	Inflammation	Angiogenesis	Fibrilation	Collagen density	Epithelialization
1	Ch-Br	+ + +	+ + + +	−	−	−	−
1	Chitosan	+ + +	+ + + +	−	−	−	−
1	Control	+ + +	+ + + +	−	−	−	−
7	Ch-Br	+ +	+ + +	+ + +	+ + +	+	−
7	Chitosan	+ +	+ + +	+ + +	+ + +	+	−
7	Control	+ +	+ + +	+ + +	+ + +	+	−
14	Ch-Br	−	+	+	+ +	+ + +	+ +
14	Chitosan	+	+ +	+ +	+ + +	+ +	+
14	Control	+	+ +	+ +	+ +	+ +	+
21	Ch-Br	−	+	+	+	+ + +	+ + +
21	Chitosan	+	+ +	+	+ +	+ +	+ +
21	Control	+	+ +	+ +	+ + +	+ +	+

formation of covering tissue along with the necrosis tissue (scabs) was observed (Fig. 5-B).

In microscopic examination of Trichromason staining (Fig. 5-C), density and regularity of collagen fibers on the 21st day was evaluated in all three groups. We observed that, the density of collagen fibers in the Ch-2% w/v Br treatment group was higher and more regular than other groups.

According to the microscopic examination, on days 14 and 21 in Ch-2% w/v Br group no residual of necrosis tissue was observed. Moreover, the complete formation of epithelial tissue was recognizable on day 21. So, it seems that Chitosan-Bromolain combination had better beneficial debridement effects than chitosan alone.

Table 3 indicates the criteria of assessing wound healing such as necrosis, density of inflammatory cells, angiogenesis, fibroblast proliferation, collagen density, and formation of epithelial tissue in different groups on days 1, 7, 14 and 21.

4. Discussion

Electrospinning is one of the most common techniques for producing nanofiber in biomedical fields. Some advantages of this method are simplicity and cost-effectiveness. Moreover, local administration of drug-loaded nanofibers can create an effective concentration of medicine on affected area and reduce the adverse effect of systemic drug delivery [25–28]. On the other hand, due to their structural similarity with extracellular matrix of normal skin, electrospun polymeric nanofibers have been considering as promising scaffolds for improving cell growth and skin regeneration. They can also be used as a delivery system for providing biologically active molecules to the wound site [29]. In recent years, chitosan nanofibers prepared by electrospinning method have been significantly considered as a new drug delivery system with several biomedical applications such as wound and burn healing [20,30]. In some studies, the effects of chitosan gel debridement on second degree burns have been investigated. It has been shown that the tissue re-epithelialization processes were completely done in the treated wounds by chitosan gel. Also the proliferation of epidermis cells has been observed and it was looked like a normal tissue [18]. Honardar et al. showed that the treatment of second degree burn wounds in rabbits with chitosan based gel resulted in no hyperpigmentation and overgrowth of epidermis. However, an increase in keratinocyte number due to papillary growth of epidermis and increase in melanocyte pigmentation and melanin in dermis were observed in control group [31].

Bromelain is a mixture of proteolytic enzymes presence in all tissues of pineapple (*Ananas comosus*). It is known as an efficient debriding agent in burn treatment and tissue regeneration [32,33]. In the first phase of wound healing, proteases from bromelain hydrolyze the fibrin clot deposited on the wound. They also digest damaged components of extracellular matrix such as collagen, elastin and laminin by proteolysis effect. This digestion induces release of growth and angiogenic factors in matrix, as well as activation of bioactive chemokines and cytokines, and processing cell to cell and matrix to cell adhesion molecules. Except the proteases, bromelain contains non-proteolytic enzyme, called Escharase, which has no analytical activity against normal proteins and cleaves glycosaminoglycan substrate and efficiently removes eschars [10,34–36]. In some studies, the effect of enzymatic debridement of deep partial thickness burns with Debrase, a commercial product containing bromelain enzyme, on wound re-epithelialization was evaluated. The outcomes showed that, burns treatment with Debrase resulted in earlier re-epithelialization and cellular proliferation, when compared with its carrier and topical antibiotic dressing alone [37–40]. In another study, the plant enzymes including bromelain and papain were loaded into chitosan gel formulation and used for treatment of burns in rats. The results showed that this formulation caused faster re-epithelialization rate, degradation of necrotic tissues, prevention of infection, and reduction of oxidation in the affected tissue than chitosan alone [41].

In this study, the chitosan-bromelain nanofibers were produced by electrospinning technique. Physicochemical characteristic of electrospinning solution and synthetized nanofibers were studied. The electrospun fiber diameters are depended on several factors such as viscosity, electrical conductivity, solution concentration, voltage, nozzle to collector distance, and feed rate. Viscosity is an important parameter in nanofiber morphology. At low viscosity, surface tension influence fiber morphology which resulted in the reduction of nanofiber diameter and formation of drops instead of fibers [42,43], while high viscosity makes it impossible to extrude the solution due to cohesive nature of high viscosity [42]. The results of our study showed that, adding bromelain into chitosan solution increased viscosity without causing any problem in electrospinning process or increasing in nanofiber diameter.

Analysis of electrical conductivity indicated that the highest electrical conductivity was belonged to Ch-4% w/v Br solution, then Ch-2% w/v Br and chitosan 2.5% respectively. Generally the electrical conductivity increases with the addition of ions, as well as dissolution of drugs and proteins in water [42]. So, it seems that adding bromelain, based on its protein structure, could increase the conductivity of chitosan-bromelain solution. Different results have been reported about the effect of conductive polymers on nanofibers diameter. By increasing conductive polymer content, higher or lower diameters have been obtained. The reason could be due to the effect of other parameters such

as concentration, viscosity, and types of polymers or using different method for preparation of nanofibers [44].

To determine the mechanical properties of nanofibers, the strength of fibers should be evaluated against tensile strength. This evaluation was characterized by Young's modulus using tensile test. Our results showed that the addition of bromelain to chitosan solution improved the mechanical properties of non-cross linked chitosan nanofibers. This increase in mechanical properties in Ch-Br nanofibers could be due to the interaction between chitosan and bromelain. However, cross linking of Ch-4% w/v Br resulted in significant reduction of tensile strength (data not shown).

The water uptake capacity is an important factor in nano-dressing for absorbing the exudates of wound surface [20]. In this study, the swelling test was carried out by soaking of nanofiber in PBS at pH 7.4. According to the results, the maximum swelling was belonged to chitosan nanofiber and the minimum swelling was related to Ch-4% w/v Br. Such reduction in swelling volume could be attributed to a more rigid network formed by inter- and intra-polymer reactions and the reduction of hydrophilic groups after crosslinking process [20]. The more reduced swelling of Ch-4% w/v Br may also be due to more beads in the nanofibers structure.

The results showed that the loading of bromelain in nanofiber did not change its activity in both Ch-2% and 4% w/v Br nanofibers.

The results demonstrated that increasing concentration of bromelain resulted in sustain release behavior. Maximum release of bromelain in PBS was observed for Ch-2% w/v Br at the first 24 h and it was around 100%, while this amount of release occurred in about 144 h in Ch-4% w/v Br. The PBS medium was used because the pH of damaged tissue is in the range of 6.5–8.5. The process of slow release could be due to the presence of some beads in Ch-4% w/v Br nanofibers. Bromelain may remain in such beads and that cause slow release of bromelain. On the other hand, the burst release of bromelain from Ch-2% w/v Br nanofiber can be considered as an advantage in burn healing process.

Our results showed that the stability of bromelain activity was preserved after the certain time (6 month). In other study by Manu Sharma et al., bromelain was encapsulated into Eudragit L 100 polymer. They showed that bromelain remained activate when loaded in Eudragit enteric nanoparticle [23].

The evaluation of cytotoxicity is important parameters for dressing materials used in burn healing applications. The biosafety of chitosan had been previously studied by *in vivo* tests and the results were indicated that, chitosan has no cytotoxicity effect and is a biodegradable polymer [45]. In this study, no cytotoxicity was observed with chitosan and Ch-2% w/v Br nanofibers and bromelain with equal concentration in nanofiber. However, Ch-4% w/v Br cross-linked-nanofibers showed the cell toxicity compared to control. This result may be due to the presence of some beads containing glutaraldehyde in this nanofibrous membrane which did not remove even after several washing with PBS.

Finally, according to the obtained results, Ch-2% w/v Br nanofiber was selected for *in vivo* studies because of the better physicochemical properties and release profile as well as low cytotoxicity.

Regarding to the results of histopathological studies on second degree burn in rats, it was found that bromelain-loaded chitosan had more effects on re-epithelialization, debridement and more reduction of necrosis compared to chitosan alone. For re-epithelialization of damaged tissues in burns, it is necessary to remove necrotic tissues. In a previous study, 35% bromelain in a lipid base could efficiently remove damaged tissue from wounds or second/third degree burns [46]. In another study, wound healing effects of bromelain nanoemulsion (NEB) (9 mg per 2 g of oil phase) and bromelain nanoemulsion in a carboxymethyl cellulose (CMC) gel were evaluated in thermally-induced burn in rabbits. After 14 days, NEB showed the highest percentage of wound contraction [14].

Nexobrid is a commercial formulation of bromelain, present as a lyophilized bromelain powder and gel vehicle. This formulation is topically applied within 15 min and for a maximum of 4 h to burn wound area, at a dose of 0.02 g/cm^2. A second application is not recommended [47,48]. In this research, the effective dose of bromelain that was used was 0.06 mg/cm^2. Therefore, it can be concluded that, loading low amount of bromelain in chitosan nanofiber can be used as a beneficial and efficient drug delivery system on burn wound healing.

5. Conclusion

Burn wounds healing have a complicated process. In this study the nanofiber formulation of Ch-Br was produced by electrospinning method. Physicochemical properties and release profile of this drug delivery system were studied and showed favorable results. The effect of Ch-2% w/v Br nanofiber on second degree burn healing was also investigated. The results suggest that the Ch-Br nanofiber accelerate the process of wound healing in animal model. So, based on appropriate results on burn injuries in animal models and good properties in drug releasing investigations, this system can be proposed as a suitable system for healing burns in human.

Declaration of Competing Interest

The authors declare no conflict of interest.

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