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Preparation and optimization of silibinin-loaded chitosan-fucoidan hydrogel: An *in vivo* evaluation of skin protection against UVB

Authors: Masood Ali karami[1],Behzad Sharif Makhmalzadeh[1,2,*], MahsaPooranian[1], Anahita Rezai[3]

[1] Department of Pharmaceutics, School of Pharmacy, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran.

[2]Nanotechnology Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

[3] Department of pathobiology, faculty of veterinary medicine, shahidchamran University of Ahvaz, Ahvaz, Iran

* Correspondence: Behzad Sharif Makhmalzadeh; Nanotechnology Research Centre, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran; Email: makhmalzadeh@yahoo.com; https://orcid.org/0000-0002-1441-3127. Tel: +98-61-33738380

Abstract

Purpose: The objective of this study was to develop silibinin-loaded hydrogel for skin protection against UVB damage.

Method: Physical grafting was used to prepare hydrogel based on chitosan-fucoidan.Then, hydrogel properties, such as swelling, drug release rates, morphology, and structure, were evaluatedto determinethe optimum hydrogel for *invivo* studies. In *invivo* experiments, the silibinin permeability parameters were investigated through normal and UV-irradiated skin, anti-inflammatory property, and antioxidant effects after application of optimum hydrogel.

Results: The silibinin completely dispersed in the hydrogel, and FT-IR results showed that silibinin reacted with the chitosan and fucoidan anddemonstrated a slow release pattern. The 50% and less than 70% of the drug-loaded on hydrogel were passed through normal and irradiated skinafter 48 h, respectively. *Invivo* studies showed the effectiveness of optimized hydrogel in preventing the production of oxidative species and H_2O_2 after UVB radiation. Histological studies have shown that silibinin-loaded optimized hydrogel can prevent the hyperkeratosis, acanthosis, and infiltration of neutrophils into the dermis by UVB.

Conclusion: Optimized hydrogel effectively reduced the inflammation mediators interleukin-22 and TNF-α, which signify tissue destruction. Therefore,silibinin-loaded hydrogel can be introduced as an effective sun-protective product.

Keywords:silibinin, hydrogel, fucoidan, chitosan, chemopreventiveagent, UVB-induced skin damage

Introduction

The human skin is the most vital aspect of the defense mechanism protecting the underlying tissues from the harsh externalenvironment.Solar ultraviolet radiation (UV) is one of the most frequentfactors that can causeserious disorders ofthe skin. UVA (315-400 nm) and UVB (280-315 nm) can reach the surface of the earth and adversely affect human health (Gonzage 2009; Cavinat et al. 2017). UVA is responsible for immediate pigment darkening, which disappears within two hours and has chronic effects similar to those of UVB (Gonzage 2009). Particularly UVB,due to its high energy,can cross the epidermis and reach the upper dermis, where it interacts with cellular chromospheres, leading to DNA damage and increased oxidative stress. Chronic exposure of the skin to UVB causes skin aging, which is characterized by skin fragility,laxity, roughness, dryness, hyper-pigmentation, blister formation, a leathery appearance, and formation of wrinkles and hyperkeratosis(Mohamed et al. 2014). Efficient skin protection methods can prevent the harmful effects of UVA and UVB.

Silibinin (SB) is a potent and principal component of silymarin extracted from Silymarianum (Milk thistle). SB is a secondary alpha-hydroxy ketone (Fig 1) and the main polyphenolic biologically active constituent found in Milk thistle (approximately 50-70%). SB has beneficial effects forinhibitingUVB-induced skin injuries(Marchiori et al. 2017).Studies have shown that the dietary feeding of silibininprovidesstrong protection against UVB-induced damagetothe skin either by (a) either preventing DNA damage or enhancing repair; (b) reducing the UVB-induced hyper-proliferative response; and (c) inhibiting UVB-caused apoptosis and sunburn cell formation, possibly via the SB-caused upregulation of p53 and p21/cip1 as major UVBdamage control sensors (Dhannalakshmi etal. 2005). Unfortunately, SB has low bioavailability due to its degradation in gastric fluid, low water solubility, and poor enteral absorption(Zhao et al. 2016).

Therefore, it seems that the topical application of SB couldbe a new opportunity for skin protection against UVB radiation.

Hydrogel is a swellable three-dimensional network formed by cross-linking polymer chains. Hydrogelscan be used fora wide range of applications, such as in controlled drug delivery systems (DDSs) and as specialized carriers for various macromolecules. Hydrogels protect drugs fromhostile environments andcan control drug release by changing the gel structure in response to environmental stimuli (Qiu& Park 2001). Hydrogel possessesstrongerspreadability and stability and exhibitsabetter drug-controlled release pattern than other semi-solid preparations. Hydrogels demonstrate more advantages as topical drug delivery systems, such as a degree of flexibility similar to natural tissue, and they act as drug depots in the skin.

Natural polymers, such as polysaccharides and proteins, have also been used as the structural material in hydrogels. This is largely due to the current interest in the intrinsic properties of these polymers, including biocompatibility, low toxicity, andsusceptibility to enzymatic degradation. Among these polymers, polysaccharides do not demonstrate disadvantages, such as immunogenicity and the potential risk of transmitting animal-derived pathogens. These attractive natural polysaccharides share the benefits of other natural polymers but do not induce an immune response.Chitosan is a very well-known polysaccharide (Fig 1) that has been used for hydrogel formation. Hydrogels composed of chitosan alone or in combination with alginate, methylcellulose, or carbopol have been used as drug delivery systems (Dai et al. 2008; Badek et al. 2000).

Fucoidan, asulfated polysaccharide (Fig 1)extracted from brown algae, was first isolated by Kylin almost a century ago and was found to contain a significant amount of L-fucose and sulfate ester groups. Recently, fucoidan has been studied extensively due to potential anti-tumor, anti-

viral, anti-complement, and anti-inflammatory activities(Bhattarai et al. 2010).Fucoidan is a sulfated polysaccharide,and its photo-protective effects against UVB have been investigated. Fucoidan inhibits UVB-induced MMP-1 expression in human skin by inhibiting the ERK pathways (Moon et al. 2008). Therefore,the novelty of this study is that a marine natural-based vehicle with sun-protective properties is preparedfor the delivery of SB as a natural UV-protective agent.In this study,a hydrogel composed of chitosan and fucoidan was used as a silibinin topical delivery system to protect the skin against UVB.

Insert fig 1. **Materials and Methods**

Materials

Silibinin, Fucoidan, chitosan, diamino-benzidine were purchased from Sigma-Aldrich, lactic acid, N-Butanol, and potassium monobasic were purchased from Samchum (South Korea), DMSO, Tris-buffer, dodecyl sulfate, and methyl green have been purchased of Merck (Germany). Trademark® sun-protective cream was provided by Cinere (Cosmetic Company, Tehran, Iran).

Methods

Experimental design and optimization

Several parameters could affect final hydrogel properties and its permeability through the UVB-irradiated skin. Therefore full-factorial design was used concerning two independent variables at three levels in this study for experimental design. Major variables in the determination of

hydrogel's properties were chitosan and Fucoidan percentage. Nine different formulations with low and high values of Chitosan percentage (1 %, 2%, and 4%), Fucoidan percentage (0/1%,0/2 %, and 0/4%), and 100 µM SBwere prepared and their properties such as porosity, hydrogel swelling ratio and morphology, drug release, and permeation parameters through mice skin were evaluated(Sezer et al. 2008). Optimization was done based on a checkpoint analysis and performed with Minitab 16 software to find the level of independent variables that would obtain a maximum value of swelling ratio after 60 min and the maximum amount of % drug release after 1 h (% R_1). Swelling ratio and % drug release are critical parameters that determine SB concentration into the skin as the main site for protection against UV-B.

Preparation of Hydrogels

Fucoidan was dissolved in a 1% w/vlactic acid solution contained 0.2% Span 20 by mechanical shaking at 300rpm for 1h. Then chitosan was added to swell in the fucoidan solution, SB with different concentration (0.048, 0.12, and 0.22 mg/g equal to 100, 250, and 450 µmol/l) added with agitation, and then 0.5% w/v glutaraldehyde was added as a crosslinker agentand left overnight to prepare hydrogels. Hydrogels were kept at 4°C (Qiu & Park. 2001). The uniformity of drug in the hydrogel was investigated by the drug content uniformity test described in the next experiment.

SB content and pH of hydrogels

1 g SB-loaded hydrogel was dissolved in 100 ml of buffer phosphate (pH= 7) including 0.3% span 20. The obtained solution was filtered through a Millipore filter (0.45 µm) and then the SB

content estimated by HPLC method (Baviskar et al., 2013).The experiment repeated triplicated and SB content reported as mean±sd.

1 g SB-loaded hydrogel was weighed and mixed with 20 ml of purified water and then the pH was measured by pH meter (Corning, USA).

Spreadability measurement

For this purpose, 1 g SB-loaded hydrogel was pressed between two plates (15×15 cm) by putting 5 g standardized weight on the upper plate. After 5 minutes diameter of the spread circle was measured and reported as spreadability value (Rao et al., 2009).

Rheology measurement

The rheology of hydrogels was performed by a cone and plate viscometer(Thermo Scientific, Germany) at 32 ℃ (diameter 20 mm; cone angle 1°). Viscosity was measured at shear rate 0.1-200 s^{-1}over 250 sec (Cuomo et al., 2019).

Swelling Study

The swelling studies were carried out gravimetrically in pH 7.4 phosphate buffer (PBS).

One gram hydrogel was put in a petri dish and 10ml of PBS (pH 7.4) was added until the hydrogels reached the constant weight at 25°C. At predetermined time intervals, the hydrogels blotted with the filter paper to remove excess water and reweighed. Each experiment was performed in triplicate. The swelling ratio (Ds) was calculated using Eq. 1.

$$Ds = \frac{Wt - W0}{Wt} \times 100 \qquad \text{Eq.1}$$

WhereWt is the weight of hydrated gel at time t, W0 is the initial weight of the gel(Qiu& Park. 2001).

Characterization of the hydrogel by FT-IR

The nature of the interacting forces can be changed during the gelation process. This can be observed with a Fourier transform infrared spectroscopy (FT-IR). FT-IR spectra were recorded on an FTIR spectrometer (Uker, Vertex70, and Germany) at room temperature. Hydrogel samples were dried under atmospheric conditions for 24 h. Chitosan, Fucoidan, SB, and the dried hydrogel with SB and without SB were triturated with KBr in the ratio of 1:100 and pressed to form pellet samples and their spectra were prepared in the range of 400–4000 cm^{-1}(Badek et al. 2000).

Differential scanning calorimetry (DSC)

The thermal properties ofSB-loadedoptimal hydrogel and pure SB were examined using DSC (Mettler DSC 30, Mettler-Toledo, OH, USA). The samples were placed in aluminum pans, and in a heating program, the temperature was increased to 200 ^{o}C (heating rate: 5 °C/ min)(Marchiori et al. 2017).

In-vitro Drug release

Drug release profiles were performed by static diffusion cells with a thermo-regulated water jacket and the temperature was maintained at 37 ^{0}C. The donor and receptor phases were separated by the acetate cellulose (Spectra/Por, molecular weight cut of 3000–4000 Da) membrane.To achieve solvent equilibrium between donor and receptor phases, the donor and receptor chambers were filled with receptor phase and left for 1 h, and then the receptor phase was removed from the donor chamber and replaced with 5 g of 100µmol/L SB- loaded hydrogel. 30 ml of buffer phosphate (pH= 7) including 0.3% Sorbitanmonolaurate (span 20) was used as the receptor phaseto obtain sink condition. Samples were picked up in determined intervals over 48hrs, and the amount of SB released was determined by the HPLC method. The percentage of drug release after 1 (%R1), and 48 h (%R48) were determined as signs of burst and sustained release. The drug release mechanism was evaluated by fitting different models and calculating the correlation coefficient (R^2). In this experiment, an aqueous suspension of SB with the same concentration was used as control. Also, an *In vitro* release experiment was performed for optimized hydrogel contained 100, 250, and 450 µmol/L SB.

The Ex-vivo skin permeation studies

Dorsal skin was separated from newly sacrificed mice with ketamine.The subcutaneous fat was completely removed by cold acetoneand stored at -20°C. the full-skin thickness was measured by digital micrometer (AAOC, France). The frozen skin was kept at room temperature to carry out the permeability experiment.An electrical conductor at 300 Hz was used for skin integrity evaluation by two stainless steel electrodes. Skin samples with the electrical resistance of more

than 3.9 KΩ/cm^2 were used in skin permeation experiments (Davies et al., 2014). The in-vitro skin permeation studies were performed using vertical glass diffusion cells fabricated in house with an effective diffusion area of approximately 1.53 cm^2. The volume of the receptor section was 10ml. The hydrated skin was mounted between the donor and receptor compartments of the cell without any damage due to the diffusion cell apparatus.The receptor medium was filled with Buffer phosphate including 0.3% Span 20 to obtain sink condition and constantly stirred using the externally driven magnetic beads at 37 °C and 300 rpm throughout the experiment. At each interval times (0.5, 1, 2, 4, 6, 8, 24, 28, 48 h), a 2 ml sample was withdrawn from the receptor medium and immediately replaced with an equivalent volume of Fresh Buffer including Span 20. The permeated amount of SB was determined using the HPLC method(Makhmal Zadeh et al. 2018).The results were plotted as cumulative permeated drug percent versus time. Based on these plots, the apparent permeability coefficient (Eq.2) and the steady-state permeation flux (J_{ss}) (Eq.3) were calculated.

$$Papp = \frac{dQ}{dt} \times \frac{1}{A.C\,0} \qquad \text{(Eq. 2)}$$

$$Jss = C\,0 \times Papp \qquad \text{(Eq. 3)}$$

dQ/dt is the steady-state appearance rate on the acceptor side of the skin. A is the area of the skin (CM^2) and C_0 is the initial concentration of the drug in the donor phase. On the other hand, the cumulative permeated amount of SB through the skin after 48 h (Q48) was calculated for all skin samples.

In-vivo UV-B protective experiments

-Animal care and treatment

The 6 to 7 week old female hairless mice used in this study were purchased from the laboratory animal center, Jundishapur University of Medical Sciences, Ahvaz, Iran. All animal experiments were carried out by the Ethical Committee of Ahvaz Jundishapur University of Medical Sciences (Approval number: N-9804). Mice were housed five per each cage and acclimatized for at least 1 week before the start of the experiment, and housed in the Animal Resource Facility of the Ahvaz Jundishapur University of medical sciences under the following conditions: 12 h dark/12 h light cycle, 24 °C temperature, and 10% relative humidity. The mice were fed a standard diet and water (Moghimipour et al. 2013).

- **UVB irradiation protocol**

The UVB irradiation was produced by Philips TL40W/12 RS lamp (Medical-Eindhoven, Holland) equipped with a UVB meter (Lutron Electronic, Taiwan) to regulate UV radiation dosage with a distance of 20 cm between the UVB lamp and the skin surface. The mice anesthetized by intraperitoneal injection of 90 mg/kg ketamine and 3 mg/kg xylazine and then received 600 mJ/cm ^{2} for 10 min, 5 times per week for 2 weeks.

– Hydrogel administration

Mice were randomly divided into 10 groups with 3-7 animals in each following groups: (1) Naïve (non-irradiated);(2) irradiated without treatment;(3) treated with blank hydrogel;(4) treated with the aqueous suspension of SB(100 μmol/L);(5) treated with hydrogel including SB(100μmol/L),(6) treated with hydrogel including SB (200 μmol/L);(7)treated with hydrogel including SB(250 μmol/L);(8) treated with hydrogel including SB (350 μmol/L); (9) treated

with hydrogel including SB(450 μmol/L) and (10) treated with Trademarke®Sun Screen (positive control). All groups were treated with 2mg/cm^3 of hydrogel or Trademarke® Sun Screen before UVB irradiation.

- **Histochemical Detection of H2O2-Producing Cells**

Immunohistochemical detection of H2O2 in all groups was done following a previously described procedure. 72 h after UVB irradiation or UVB irradiation plus hydrogel mice were euthanized and 6-cm-thick skin sections were incubated with 0.1 mol/L Tris-HCl buffer (pH 7.5), containing 1 mg/mL glucose and 1 mg/mL diaminobenzidine for 2 h at 37°c. Sections were then washed in distilled water and counterstained with methyl green and then identify H2O2 producing cell with a fluorescent microscope (Gonzage 2009).

- **Assay for Lipid Peroxidation products**

The epidermal microsomal fraction was used to determine the epidermal lipid peroxidation level using the thiobarbituric acid reaction method, as described previously (Sharma et al. 2007). 400 μM SB hydrogel and blank hydrogels were applied on the skin before UVB exposure and then 24 h after the UVB irradiation, mice were sacrificed and skin samples were collected. Briefly, 0.2 mL of the microsomal fraction was treated with 0.2 mL of 8.1% sodium dodecyl sulfate (SDS) and 3 mL thiobarbituric acid. The total volume was made up to 4 mL with distilled water and kept at 95 ^{o}C in a water bath for 1 h. The color was extracted with n-butanol. The absorbance was measured at 530 nm, and the resultant lipid peroxidation was expressed against control (normal skin).

- **Histologic evaluation of the skin**

For histologic evaluation, 72 h after UVB irradiation or UVB irradiation plus hydrogel, mice were euthanized and the skin tissues were fixed in 10% formaldehyde in 0.1M phosphate buffer (pH 7.4) overnight and fixed in paraffin. Sections were stained with hematoxylin-eosin and Masson'strichrome stain to identify collagen fibers and the inflammatory cellular response with 20× and 40× objectives. The thickness of the prickle cell layer was measured using an optical microscope (Olympus BX 51; Olympus).

- **Detection of pro-inflammatory cytokines in skin sections**

72 h after UVB irradiation or UVB irradiation plus hydrogel mice were euthanized and after the addition of phosphate-buffered saline, skin tissue samples were homogenized with a homogenizer (ULTRA-DISPERSER Type T25-S2; Janke& Kunkel GmbH & Co. KG IKA-Labortechnik) and an ultrasonic processor (Model VP-5 T; TAITEC) for a short time while cooling on ice, and samples were then centrifuged for 15min at 10000 × g at 4°C. The supernatant was stored at − 70°C until use.IL-22 and TNF-α levels in the skin tissue were then quantitatively measured using ELISA kits (Endogen) with a detection limit of < 5 pg/mL (Maruyama et al. 2015).

Silibinin assay method

The determination of the amount of SB was carried out by HPLC with a C_{18} column (Waters; 25 cm, 5 µm diameter) using a UV detector at 288 nm and methanol/water (80/20) as mobile phase

at a flow rate 1 ml/min. The limit of quantification (LOQ), accuracy, repeatability, and linearity were evaluated as method validation. The injection volume was 50 µL.

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Data analysis and statistics

All the experiments were repeated three times and data were expressed as the mean values. Statistical data were analyzed using the one-way analysis of variance (ANOVA) and $P < 0.05$was considered to be significant with a 95 %confidence interval. To figure out the relationship between the dependent and independent variables, the simultaneous multi-regression test was used.

Results and Discussion

Validity of the drug measurement method

Regression analysis showed a significant relationship between concentration and area under the curve (P= 0.001) within the concentration range of 0.001-50 mg/ml and with acorrelation coefficient of R^2 =0.999. The LOQ was 0.001 mg/ml, and all reported concentrations were above this LOQ. The lack-of-fit, which appeared in the estimated absorbance changes, was not significant (P= 0.116). The accuracy of the assay method indicated the maximum difference between the reported concentration values and the actual values. The method showed the desired repeatability within and between days.

SB content and pH

The amounts of drug content in all hydrogels rangedfrom 97.5% to 101.6% ± 4.4% (n=3). The pH values of the hydrogels ranged from 5-5.5. These values didnot change during hydrogel storage.

Hydrogel spreadability

The spreadabilityresults are shown in Table 1. The maximum spreadability was 10.5 cm,provided by Formulation 6. A significant correlation (P = 0.029) was found between % swelling and spreadability. This means that hydrogel spreadability is primarily affected by swelling properties.

Swelling properties of hydrogels

The swelling ratios recorded after 60 minutes for all hydrogels are presented in Table 1. Significant inverse(P = 0.01) and direct (P = 0.014) correlations were found between the swelling ratio and fucoidan and chitosanconcentrations, respectively.

The maximum swelling ratio wasprovided by Formulation 3,which produceda high percentage of chitosan and a low percentage of fucoidan.The amount of water absorbed by a hydrogel is mainly dependent on hydrophilic groups in the chemical structure of the polymer, cross-linking density, and environmental conditions, such as pH (Ahmadi et al. 2015).

Chitosan is prepared by the partial deacetylation of chitin. Chitin has poor aqueous solubility because of the high number of acetylated groups and a rigid crystalline structure (Khor & Lim 2003). Therefore, chitosan that is prepared by the deacetylation of chitin demonstrates good

aqueous solubility (Murakami et al. 2010). Cross-linking the chitosan and glutaraldehydedecreases the aqueous solubility and increasesthe strength of the hydrogel.

Fucoidan is a sulfated polysaccharide with morehydrophilicity than chitosan. Unlike the hydrophilic nature of fucoidan, the lower swelling ratio was provided by hydrogels made with more fucoidan. This finding conflictswith what Sezer et al.found. They reported that the addition of fucoidan, which is more hydrophilic than chitosan, increased the swelling ratio of gels. It seems that hydrogel cross-linking withglutaraldehyde changed the swelling behavior in the present study. The swelling ratio is mainly dependent on hydrogel strength, and the hydrophilicity of the polymer didnot show a strong impact on the swelling ratio. The swellingratioprovided in the present study after one hour is equal to the swelling ratio reported by Sezer et al. after six hours.

***In vitro* SB releasedfrom the hydrogel**

Percentages of cumulative SB released after 1 h and 48 hare presented in Table 1. According to these data, all hydrogels had a rapid release, with18-26.5% of the drug released within the first hour. The highest and lowest release rates were reported forFormulations 3 and 9, respectively. On the other hand, 40-61% of the loaded drug wasreleased within 48 h; the highest and lowest values in this regard were associated withFormulations 1 and 9, respectively.

Previously, it was reported that 80-90% of loaded SB in hydrogels based on pluronic 127 and caprolactone-polyethylene glycol released after 24 h (MakhmalZadeh et al. 2018). It seems that hydrogel based on chitosan-fucoidan released SB at a lower rate,indicating that it can act as a

sustained release carrier for topical delivery of SB. Sustained release carriers providea release controlling system and possess a skin permeation controlling dosage form.

However, hydrogels generally release drugs at a sustained and slow rate. The percentage of fucoidan had a significant and reverse effect on the percentage of release after half an hour (P = 0.036) and 48 h (P = 0.001). This means that increasing the percentage of fucoidan reduces the drug release rate. However, the effect of chitosan percentage on release was not significant. Therefore, by changing the percentage of fucoidan, the desired release profile can be obtained.

The drug release kinetics were evaluated by fitting the release data to zero-order, first-order, Hixon-Crowell, and Higuchi models. The results showed that the Higuchi model (R^2= 0.974) was the best for describing the mechanism of release.The Higuchi model is applied to describe the rate of drug release from a matrix (such as a hydrogel) such that the solvent causes the hydrogel to gradually swelland the concentration gradient is linear from the saturated concentration of the drug in the hydrogel matrix to the concentration at the hydrogel-dissolution medium interface (Mircioiu et al. 2019). Therefore, SB provided a saturated concentration in the hydrogel, and the release rate was mainly controlled by hydrogel swelling.

Insert Table 1

Optimized hydrogel composition and characterization

Based on the significant correlation between% chitosan and % fucoidan with swelling ratio and $\%R_1$, the optimized hydrogel should be made of4% chitosan and 0.1% fucoidan.However, this hydrogel presented instability during storage, and for this reason,the optimized hydrogel was

made of 3% chitosan and 0.15% fucoidan. The characteristics of the optimized hydrogel, such as thermal behavior, rheology, morphology, FT-IR spectrum, *in vitro* drug release, permeability through normal and UV-treated mouse skin, and its*invivo*sun protection efficacy, were evaluated.

- **DSC of optimized hydrogel**

The thermal behavior of the optimized hydrogel and pure SBin the heating programis illustrated in Fig2. In this heating program, the SB and SB-containing hydrogel were heated to a temperature tanging from0°C to 200°C, and the corresponding thermograms were prepared. SB has a melting point in the range of 164-174°C (Pooja et al. 2014), which is highly broadened in an optimum hydrogel thermogram. This indicates that the SB was homogeneously dispersed in the hydrogel, which mightdue to the lower amount of SB in hydrogel compared to pure SB. Alternatively, it might be due to the low packing density of hydrogel aspreviously reported insilibinin-loaded nanoparticles (Sahibzada et al. 2017). The thermogram of the optimized hydrogel showeda major phase transition around 120°C that seemed tocorrespond to the ordered structure of chitosan-fucoidan hydrogel. Chitosan hydrogel demonstrated a glass transition temperature of around 130°C (Ray et al. 2010) that changed to an ordered structure in the chitosan-fucoidan hydrogel prepared in the present study. This ordered structure helps to release SB more quickly than low ordered hydrogels.

Insert Fig 2

Rheology of optimized hydrogel

The rheogram of the optimized hydrogel is shown in Fig3. This hydrogel demonstrated shear thinning behavior and a Newtonian region at low values of the shear rate. It seems that the

chitosan-fucoidan interaction provided a high viscosity in the shear rate near zero,meaning that the aqueous phase didnot flow at rest and the gel network was formed by chitosan and fucoidan.

Insert Fig 3

Evaluation of optimized hydrogel by FT-IR

Chitosan presents aband at 1559.17 cm^{-1},which is assigned to the stretching vibration of the amino group of chitosan and 1333.5 cm^{-1} according to the vibration of C-H (de Souza et al. 2009).The disappearance of the 1595 cm^{-1}band in the hydrogel indicates cross-linked chitosan that results in the formation of gels (Fig 4). On the other hand, band 1240 cm^{-1} belonging to the fucoidan sulfate group has also disappeared, which seems to be involved in the gelation process as well. SB has three specific bands at 3457 (OH group), 2946 (CH group), and 1508 (aromatic ring)(Sezer et al. 2013), all ofwhich disappeared in the hydrogel. This finding indicates that there is a bond between SB and hydrogel, which may be a reason for the relatively slow release of the drug.

Insert Fig 4

-*In vitro*SB release through the optimized hydrogel

The hydrogel contained 100, 250, and 450 µmol/L SB as demonstrated by the release profiles in Fig 5. The results indicated no significant difference ($P>0.05$) between SB release profiles from hydrogels loaded with 100, 250, and 450 µmol/L SB. This means that SB concentration did not affect the release amount and mechanism. It seems that the SB in three hydrogels provided similar gradient concentrations. Based on this finding, the SB in three concentrations

demonstrated saturated solubility in hydrogels. The zero-order is the best model of SB release through hydrogels. It means that swelling may control SB release.

Insert Fig 5

-Permeability of SB loaded in optimized hydrogel through normal and UV-irradiated mouse skin

All skins were hydratedaround 20-30% before the test was started. Therefore, all e skins had a close degree of hydration. The thickness of the skin samples was 320±14 µm.The permeability parameters, including flux and the cumulative permeated amount of SB after 48 h (Q48),were calculated (Table 2). Previously, theJss of silibinin-loaded in PolyGelTM gel and Pluronic F127 gel through normal mouse skin werereported to be 0.8 and 0.25 µg/cm^2 h, respectively (MakhmalZadeh et al. 2018).These valuesare comparable to thefindingsofthe present study. Based on these findings,the following conclusionsare presented:

- The penetration of irradiated skin increased compared to normal skin, indicating damage to the skin structure. UVB radiation is a well-known initiator of inflammation responses mediated by the skin that is associated withthe production of reactive oxygen species (Ishida &Sakaguchi 2007). UVB decreases the expression of loricrin, involucrin, and filaggrin,which play an important role in the skin barrier (Kim et al. 2017). In another study, it was reported that the combination of UVB radiation and oxidative stress impaired the skin'smolecular transport barrier after 5 h of radiation (Hernandez et al. 2019).

- Optimized hydrogel significantly reduced drug passage through the skin compared to the aqueous control. This reduction means that more of the drug stays in the skin. We found that 40-60% of loaded SB was released after 48 h through hydrogels. These values are greater than the values of Q48 provided by the optimized hydrogel through normal and UV-treated skins. This means that the rate of drug release is faster than the rate of SB permeability through different skins. Therefore, the drug release is not a rate-limiting step in SB permeability. Hydrogel decreased Q48 compared with the control whenthe skin's resistance and barrier against SB were strengthened. It seems that the hydrogel decreased SB skin permeability by increasing skin hydration and decreasing SB solubility in the skin.

SB,as a sun-protective substance,should stay on the epidermis to prevent sunlight from damaging the skin. It seems that the hydrogel has been able to retain silibinin in the skin. In a previous study, SB flux from rat skin was reported to be 0.43 $\mu g/cm^2.h$ (Pooja et al. 2014),which is in agreement with the results of this study.

Insert Table 2

Pathological results

The histological changes in different skin sections affected by UVB are presented in Fig 6. The first notable change was the increase in the thickness of the epidermis due to the proliferation of spiny cells called acanthosis. Also, hyperkeratosis (increased keratin layers) was another change that was visible on the surface of the epidermis. Within the epidermis, cells with wrinkled, dark, and cytoplasmic nuclei are more abundant than other lateral cells called sunburn cells. In the dermis, the infiltration of inflammatory cells with hyperemia and disruption of collagen fibers wasalso observed. Dermal blood vessel dilation and vascular hyper-permeability werealso found.

These changes were previously reported in psoriasis lesions (Katayama 2018) and UVB-treated skin (Sharma &Katiyar 2010). These changes were also observed in groups receiving blank hydrogelandapositive control group.

In the treatment group with the optimized hydrogel, epidermal hyperplasia, vascular hyper-permeability, erythema, cutaneous edema, and other changes induced by UVB irradiation were reduced in a dose-dependent manner. In these groups, the lowest changewas observed in the group treated with 450 µM/L SB. Skin thickness and sunburn cell count in different groups when evaluated at 24 h after UVB exposure are presented in Fig 5. UVB irradiation increased skin thickness compared to normal skin. All UVB-irradiated skin treated with different formulations decreased skin thickness compared to UVB-irradiated untreated skin.

No significant difference was found between the thickness of UVB-irradiated skin treated with blank hydrogel, hydrogel containing 100 µM SB, and 100 µM aqueous suspension of SB. Thismeans that blank hydrogel demonstrated a protective effect against UVB irradiation damage. No significant difference was found between the skin thickness in normal skin and irradiated skin treated with a hydrogel containing 450 µM. In a previous study, hydrogel containing SB-loaded pomegranate oil-based nanocapsulesexhibited anti-inflammatory effects on skin damage induced by UVB radiationin mice (Marchiori et al. 2017). They reported that 1mg/ml SB loaded in hydrogel (around 2000 µM) reduced the skin thickness by 64-79% when compared to irradiated skin. In the present study, 450-µM SB decreased skin thickness by 100% when compared to irradiated skin. It seems that hydrogel composed of chitosan and fucoidan fortified the SB as a UVB-protective agent. Normal skin structure was observed in the control group. According to obtained results, the optimal hydrogel has been able to prevent the harmful effects

of UV-B light on the skin while preventing the morphological changes of the skin due to UVB irradiation dose-dependently.

Insert Fig 6

Determination of lipid oxidation products

Microsomal lipid fractions were used to evaluate lipid peroxidation in the epidermis. One of the symptoms of UVB-induced oxidative stress is lipid peroxidation. The UV absorption values that are a sign of lipid peroxidation were determined in different groups.UVabsorption in irradiated skin treated with450 µM SB-loaded hydrogel was 0.15±0.01,whichwas significantly lower than untreated irradiated skin (UVabsorption: 1.66 ±0.13). Irradiated treated skin with blank hydrogel demonstrated UVabsorption of 0.68±0.04, which was significantly lower than untreated irradiated skin.

H_2O_2 production in UVB-irradiated skins

H_2O_2 production was determined as a marker of oxidative stress in the skin after UVB irradiation. To demonstrate that the UVB-induced infiltration of leukocytes was a major source of oxidative stress, we localized H_2O_2- producing cells via animmunostaining process(Fig7). As shown by the immunostaining, UVB- induced infiltrating cells are the major source of H_2O_2 production. H_2O_2 cells were seen in both the epidermis and dermis by dark spots. These H_2O_2 cells were not observed in normal skin, and UVB irradiated skin was treated by 450 µM SB hydrogels.

The SB-loaded hydrogel inhibited oxidative stress and production of H_2O_2, as well as total reactive oxygen species (ROS) by epidermal cells, which activate various cell signaling pathways. This intercellular signaling playsan important role in cell growth, differentiation, and proliferation, whichcan lead to skin cancer (Pal et al. 2015).

Insert Fig 7

SB hydrogel inhibits UVB-induced inflammation

Recent studies have illustrated the pathogenic role of IL-22, a cytokine produced by NK and $CD4^+$ cells in skin disease. IL-22 was increased in human keratinocytes after UVB radiation and promoted keratinocyte proliferation and cytokine production during UVB-induced skin inflammation (Kim et al. 2017). TNF-α secretion from UVB-irradiated keratinocytes and fibroblasts was reported. Not only is TNF-αassociated with epidermal and systemic inflammatory responses, but it also promotes apoptosis, lymphocyte activation, and hyperproliferation skin disorders (Bashir et al. 2009). Therefore, TNF-α and IL-22 were measured to evaluate the protective effect of SB hydrogelagainst skin damage induced by UVB.

UVB promoted the levels of TNF-α and IL-22 significantly ($P<0.05$) when compared to non-irradiated skin (Fig 8). Applying the 450-µM SB hydrogel decreased the levels of TNF-α and IL-22 when compared to irradiated untreated skin. The level of IL-22 in irradiated skin treated with SB hydrogel didnot show a significant difference with non-irradiated skin. Blank hydrogels decreased the levels of TNF-α and IL-22 by more than 40% when compared with irradiated untreated skin. Therefore, SB demonstrated very potent anti-inflammatory effects and can inhibit damage due to UVB irradiation.

The anti-inflammatory effect of SB is affected by its carrier. In aprevious study, 2000 μmol/L of SB was loaded in nanocapsulesand dispersed into the hydrogel reduced mouse ear edema and leukocyte infiltration induced by UVB 24 h after treatment (Marchiori et al. 2017). However, SB loaded in chitosan-fucoidan hydrogel demonstrated anti-inflammatory effects with a lower concentration (450 μmol/L), perhapsdue to the anti-inflammatory effect of the blank chitosan-fucoidan hydrogel.

Insert Fig 8

Conclusion

The mixture of chitosan and fucoidan is a suitable base for hydrogel preparation, as itdemonstrated a UVB-protective effect and favored drug storage and spread on the skin. SBexhibitedUVB-protective, anti-inflammatory, and antioxidant effects. Therefore, loadingchitosan-fucoidan hydrogel with SB can produce a protective formulation that can prevent keratinocyte proliferation and differentiation, inflammation in the epidermis by decreasing inflammation mediators, such as TNF-α and IL-22. Overall, SB can be developed as a natural chemopreventive agent that can be used for the treatment of UVB-induced skin inflammation. Hydrogels composed of chitosan and fucoidan with chemopreventive effects can boostthe UVB-protective effect of SB.

Compliance with Ethical Standards

-*Conflicts of Interest*

The authors declare that they have no conflict of interest.

- *Statement of Human and Animal Rights*

The animal studies were performed after receiving approval of the Institutional Animal Care and Use Committee in Ahvaz Jundishapur University of Medical Sciences (Approval number: N-9804).

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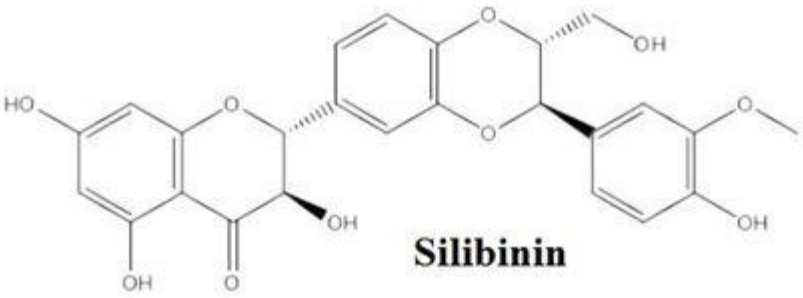
Table 1. the composition and properties of hydrogels prepared based on the factorial design (mean±SD, N=5)

formulaNo.	% chitosan	%fucoidan	% Swelling (60)	% R_1	% R48	Spreadability (cm)
1	1	0.1	23.5±3.1	25.2±1.6	60.9 ± 3.3	4.67±0.5
2	2	0.1	124.2±9.4	24.1±1.9	56.7 ± 4.5	8.7±0.66
3	4	0.1	132.3±11.3	26.9±0.9	58.1±4.2	9.8±1.1
4	1	0.2	52.4±5.3	20.3±0.7	49.3±3.8	6.2±0.33
5	2	0.2	60.7±4.8	19.3±1.1	47.8±5.1	6.7±0.40
6	4	0.2	119±7.5	24.6±0.9	50.1±3.3	10.5±0.75
7	1	0.4	29.6±3.4	20.1±0.6	43.7±4.4	5.2±0.42
8	2	0.4	39.2±2.2	21.9±1.2	44.1±2.8	5.5±0.51
9	4	0.4	70.6±8.5	18.2±0.8	40.7±4.4	7.1±0.53

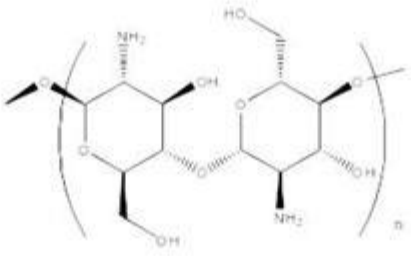
Table 2. SB permeability parameters through normal and UVB-Irradiated skins after topical application of optimized hydrogel and SB aqueous control (Mean ± SD, N= 5)

Skin Type	Flux (µg/ cm^2. h)	Q 48 (µg/cm^2)	P-value
Normal skin treated by optimized hydrogel	0.4± 0.01	21±1	0.03*
Irradiated skin treated by optimized hydrogel	0.6±0.01	29±2	-
Normal skin treated by aqueous control	0.7± 0.02	34±3	0.042**
Irradiated skin treated by aqueous control	0.78±0.02	38±4	0.035** *

*, **, ***: Irradiated skin treated by optimized hydrogel compared with: Normal skin treated by optimized hydrogel, Normal skin treated by aqueous control, Irradiated skin treated by aqueous control, respectively.



Silibinin



Chitosan

Fucoidan

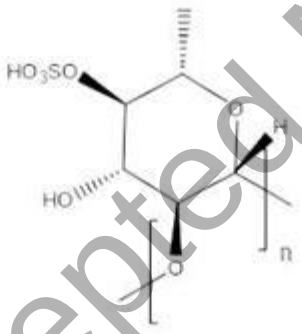


Figure 1. The chemical structure of Silibinin, chitosan, and fucoidan

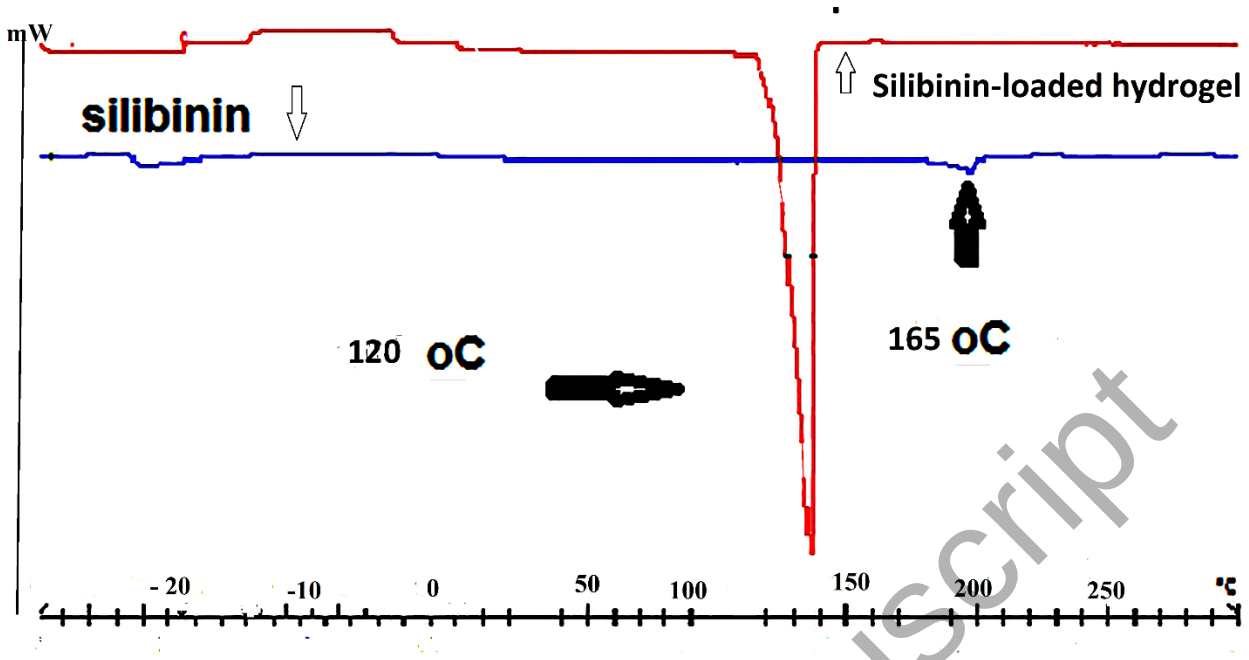


Figure 2. Thermograms of SB, and SB-loaded hydrogel obtained in the heating program

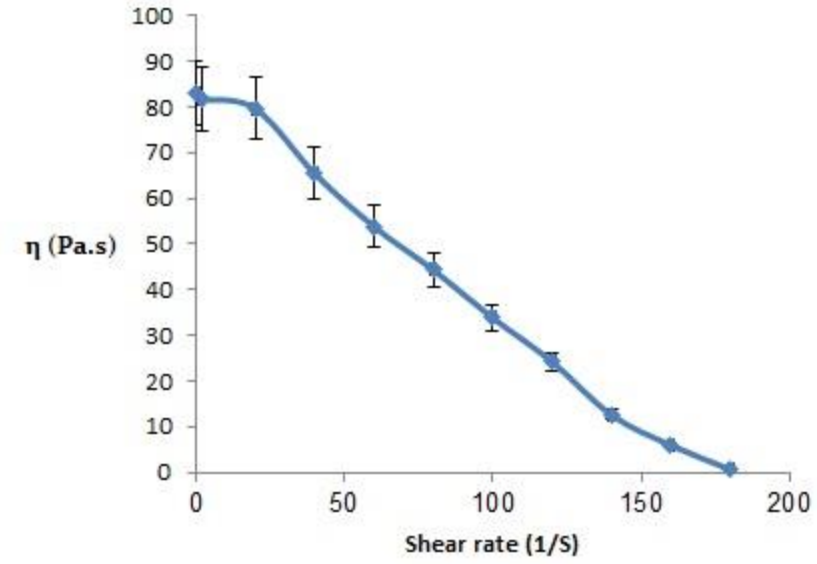


Fig 3. The shear rate-viscosity curve of optimized hydrogel

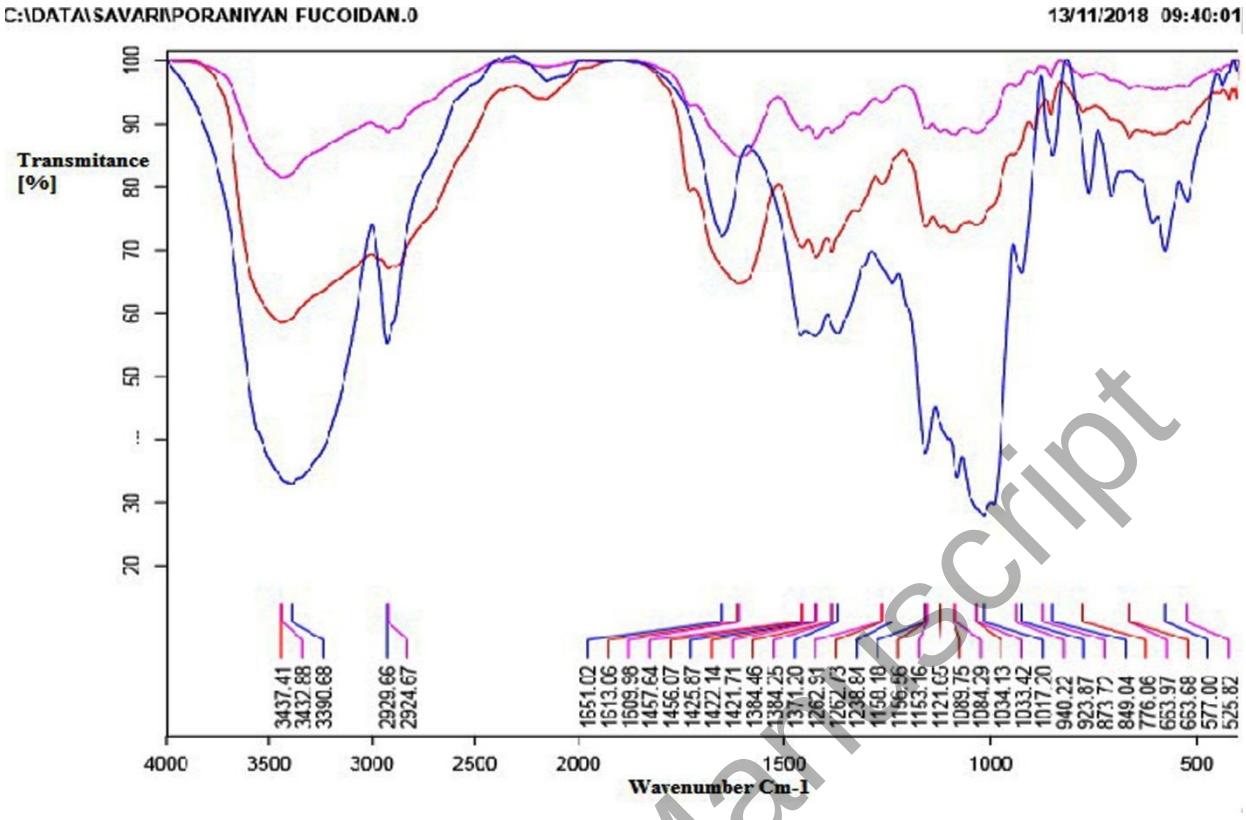


Figure 4. The FT-IR spectrum of fucoidan (blue) –blank hydrogel (red) -hydrogel with silibinin (purple)

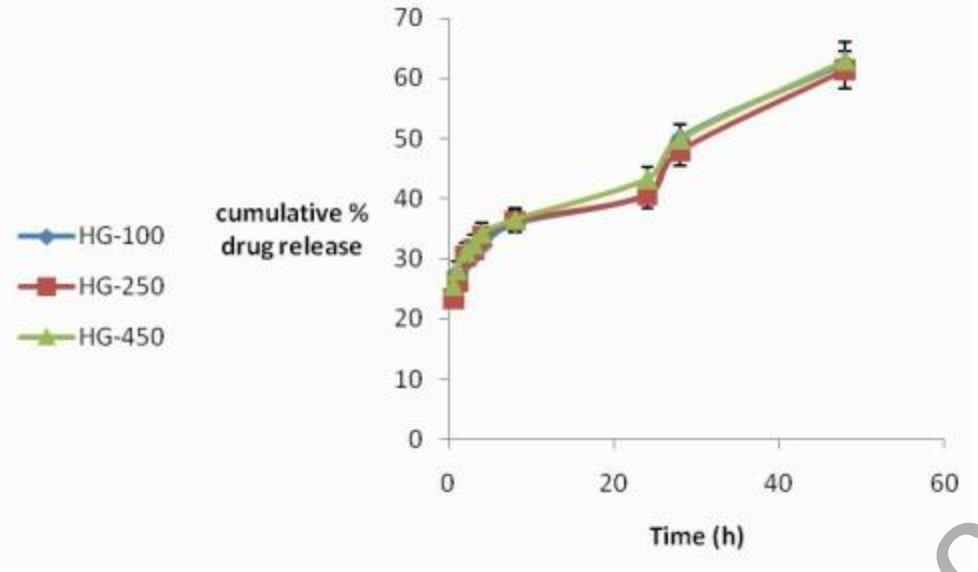
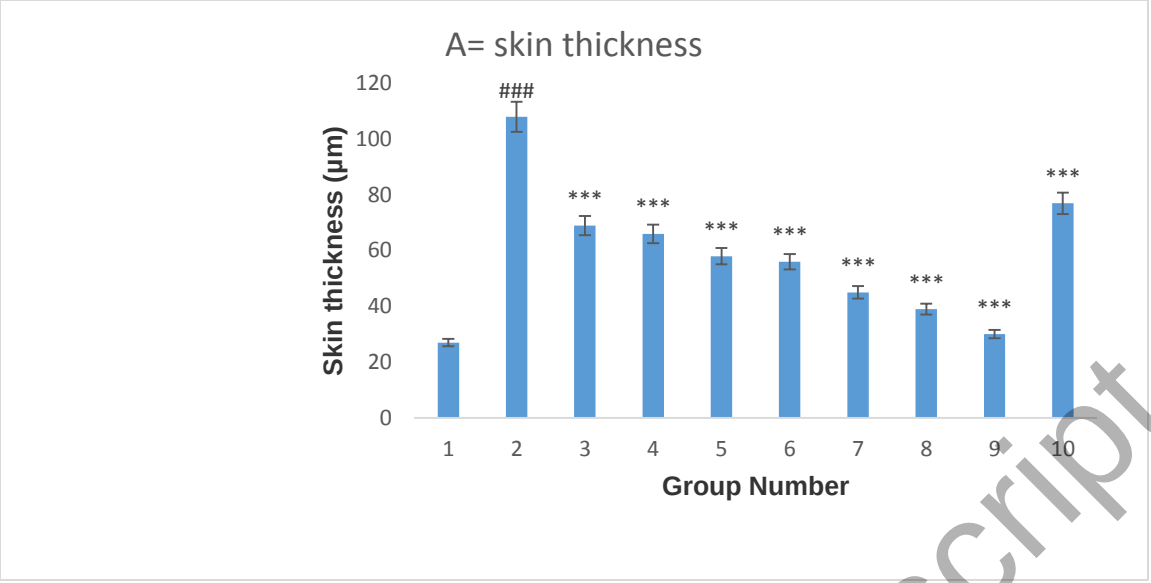


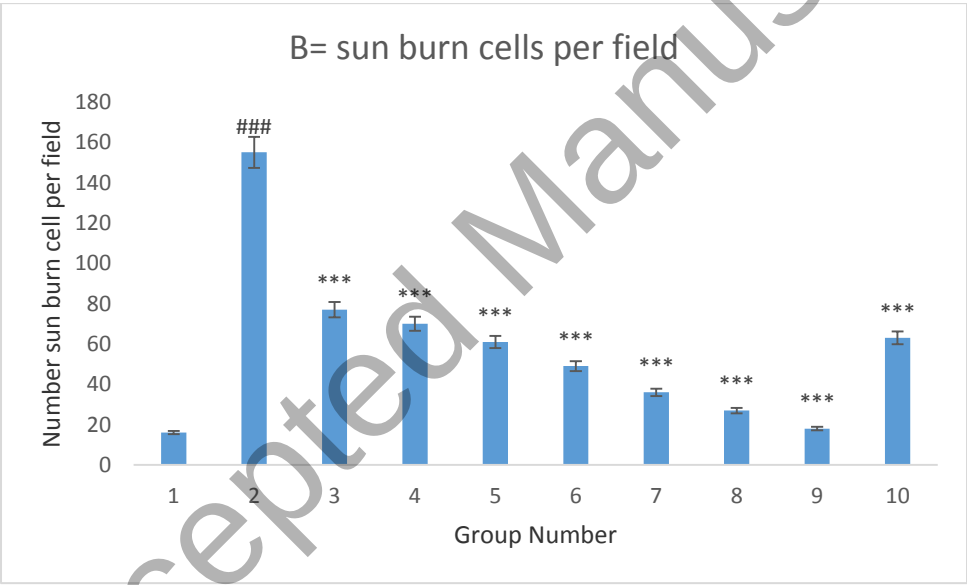
Figure 5. the release profiles of SB-loaded in hydrogel with three different concentrations (n=5)

HG-100: 100 µmol/L SB loaded hydrogel

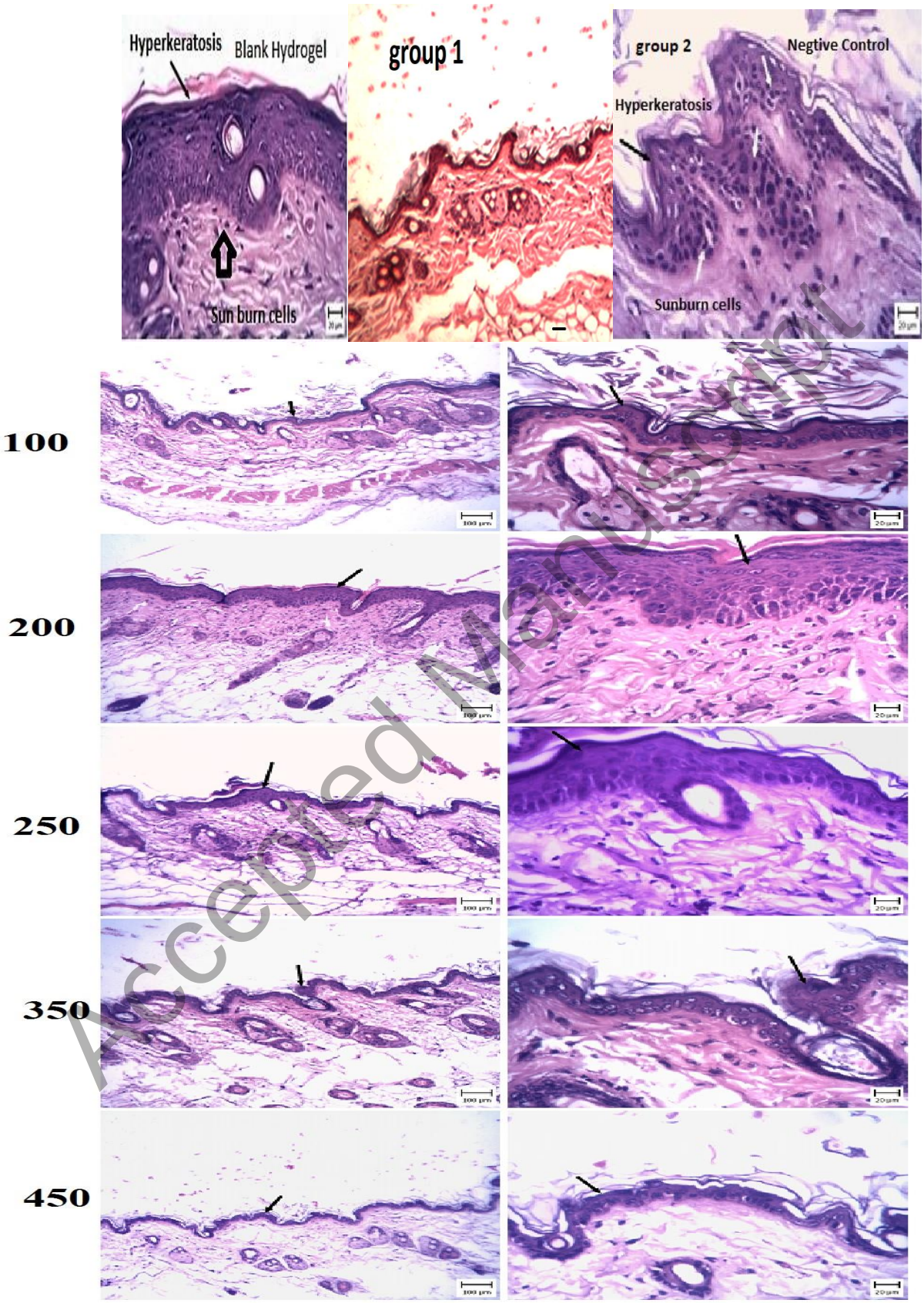
HG-100: 250 µmol/L SB loaded hydrogel

HG-100: 450 µmol/L SB loaded hydrogel





C: Histological images



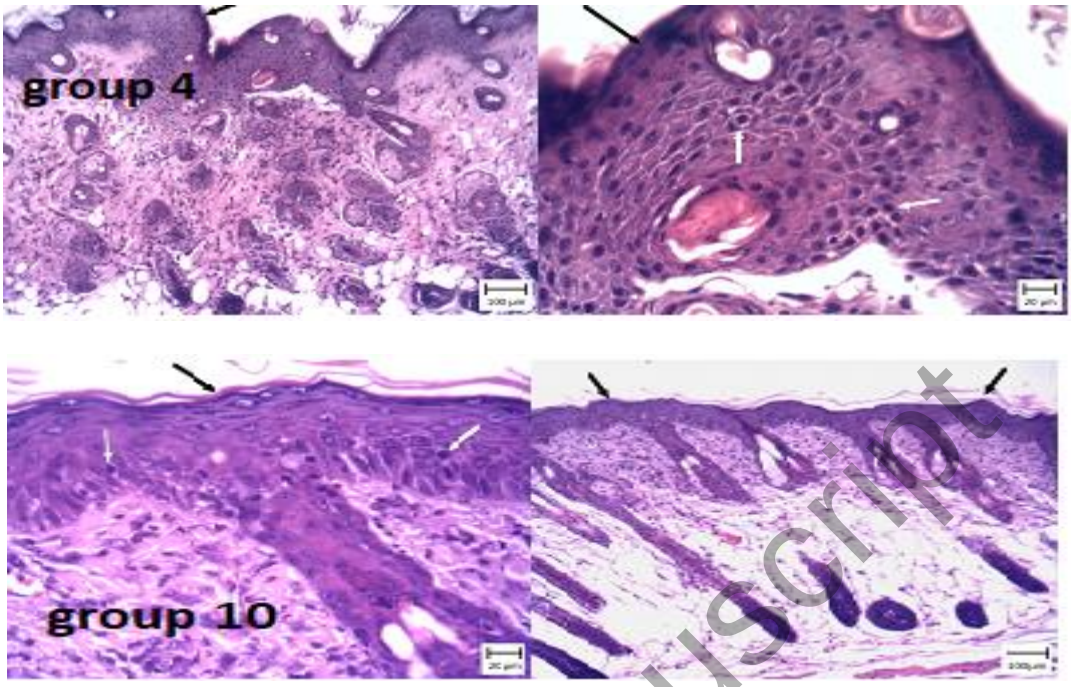


Figure 6. Protective effects of different groups on UVB irradiation-induced skin injury, A: Skin thickness ; B: Number of sunburn cells per field, C: Histological images. 10 mg/cm^2 of all formulations were applied before UVB expose to the skin and the skin thickness and sunburn cells were measured 24 h after the UVB irradiation; ### $p<0.001$ when compared with the normal un-irradiated skin (group 1), * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ when comparedwith the untreated irradiated group (group 2). 1: Normal un-irradiated skin, 2: untreated irradiated skin, 3:irradiated skin treated by blank hydrogel, 4: irradiated skin treated by 100 µM SB aqueous suspension, 5: irradiated skin treated by 100 µM SB hydrogel, 6: irradiated skin treated by 200 µM SB hydrogel, 7: irradiated skin treated by 250 µM SB hydrogel, 8: irradiated skin treated by 350 µM SB hydrogel, 9: irradiated skin treated by 450 µM SB hydrogel, and 10: irradiated skin treated by Trademark sunscreen (positive control)

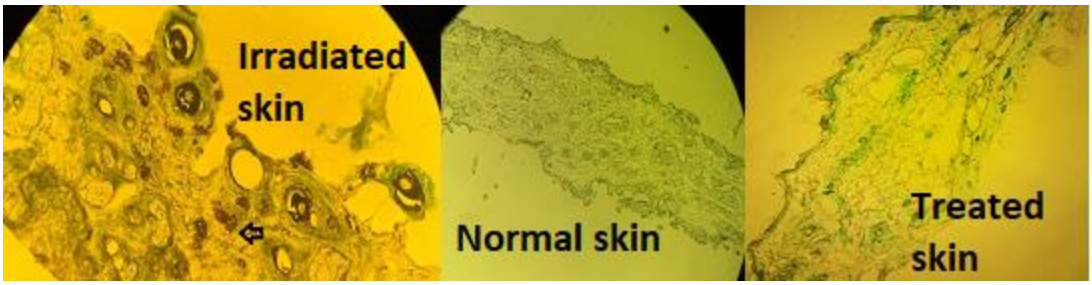


Figure 7. The production of H_2O_2 in different skins

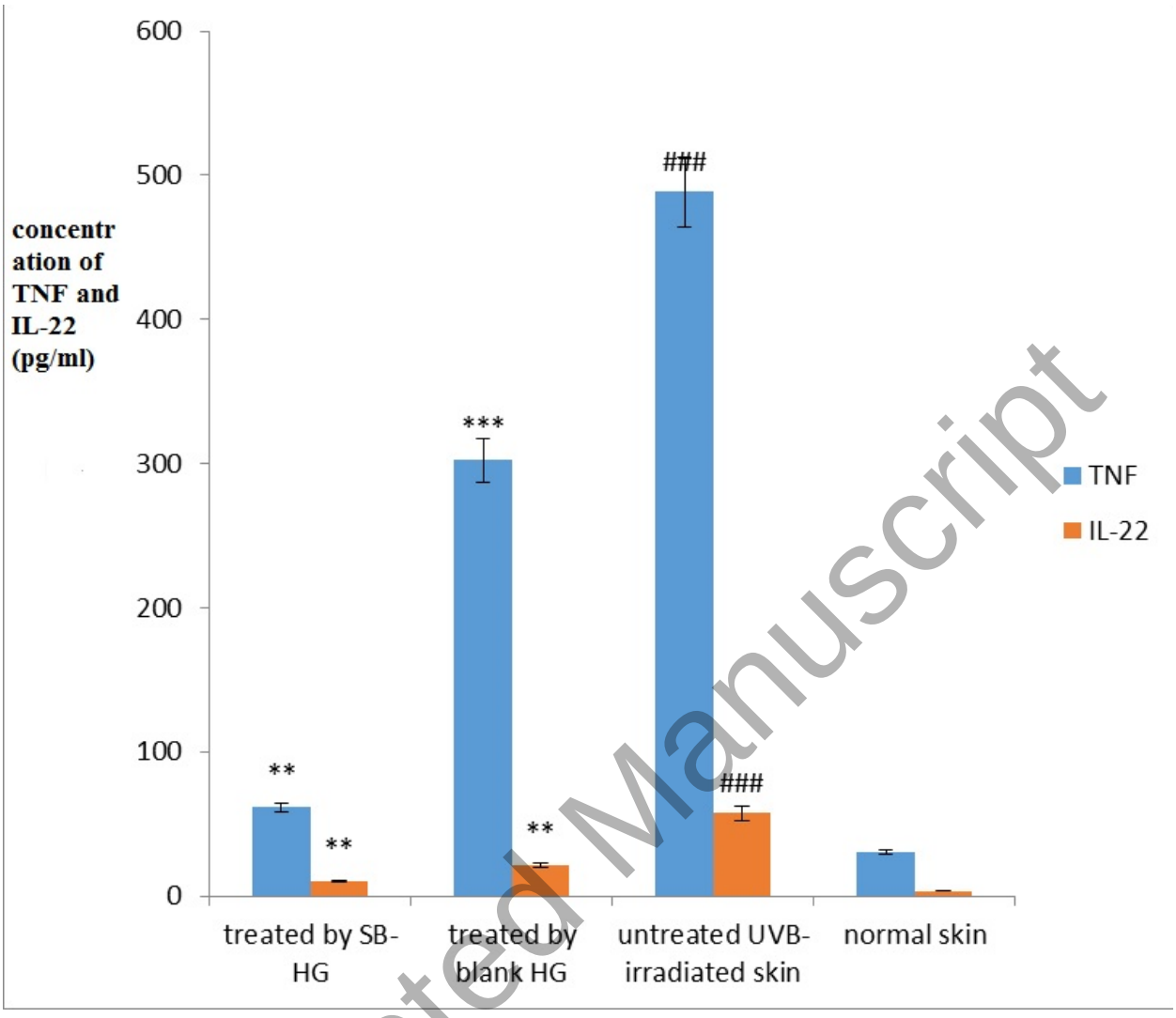


Figure 8. The levels of TNF-α, and IL-22 in different skins**.** ### $p<0.001$ when compared with the normal un-irradiated skin (group 1), * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ when compared with the untreated irradiated group **(the number of replicates was 4)**