



Chitosan/Alginate Porous Scaffolds Incorporating Silibinin-Loaded Chitosan Nanoparticles for Tissue Engineering

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Received: October 25, 2025; **Revised:** January 15, 2026; **Accepted:** January 25, 2026; **Published:** April 8, 2026

Abstract

Background: Silibinin, a plant-derived compound with known biological properties, has been studied for its role in cell differentiation, particularly in stem cells.

Methods: This experimental study designed a drug delivery system using chitosan nanoparticles loaded with silibinin (SCNPs) in alginate/chitosan (Alg/Chi) scaffolds to enable sustained, prolonged silibinin release. The scaffolds were investigated using physical, mechanical, cellular, and microbial analyses.

Results: The addition of nanoparticles affected the scaffold properties, including tensile stress, swelling behavior, and porosity, due to nanoparticle impregnation. These scaffolds showed good biocompatibility and antibacterial activity against *E. coli* and *B. cereus* bacteria. The system, featuring optimal Silibinin release, created a highly interconnected 3D porous microstructure that promoted the attachment and growth of rat bone marrow-derived mesenchymal stem cells. In addition, the Chi/Alg/SCNPs can be considered as a suitable scaffold for tissue engineering applications due to their outstanding physicochemical properties.

Conclusion: Incorporating silibinin-loaded chitosan nanoparticles (SCNPs) into chitosan/alginate porous scaffolds improved their physicochemical and mechanical properties and enhanced rat bone marrow-derived mesenchymal stem cell attachment. Therefore, Chi/Alg/SCNP scaffolds represent a promising platform for tissue engineering applications.

Keywords: Chitosan/alginate scaffold, Chitosan nanoparticles, Silibinin, Sustained release, Stem cell studies

Introduction

Silibinin, the principal bioactive constituent of silymarin derived from milk thistle, belongs to the flavonolignan class of compounds. It has exhibited notable anticancer activity across a wide range of tumor types and has been employed in combination with various chemotherapeutic

drugs to enhance their therapeutic effectiveness. Previous studies have shown that silibinin can promote differentiation of hair follicle stem cells into neural-like cells (1, 2). Moreover, silibinin modulates cancer stem cell properties by suppressing stemness while promoting cellular differentiation and apoptosis, thereby exerting



significant antitumor activity in breast cancer stem cell models (3). Silibinin possesses a hydrophobic, polycyclic structure; however, its poor aqueous solubility limits its bioavailability and, consequently, its clinical applicability (1, 2). Despite its promising biological properties, silibinin's clinical translation is substantially constrained by its limited aqueous solubility and low bioavailability, which hinder efficient delivery to target tissues. Consequently, optimizing the bioavailability and delivery of hydrophobic compounds remains a critical challenge. In this context, nanocarrier-based systems, including nanoparticles and nanomicelles, have been developed to enhance the delivery and therapeutic performance of hydrophobic drugs (4). Previous studies have demonstrated that incorporating nanoparticles into stem cell culture systems can effectively modulate a range of stem cell behaviors (5). Similarly, polymeric nanoparticles increased cellular clustering and lineage-specific gene expression (5). In parallel, three-dimensional (3D) scaffold-based systems have emerged as a cornerstone in tissue engineering (6). Mesenchymal stem cells have attracted attention in tissue engineering because of their ease of isolation and their ability to differentiate into cell types (7). Chitosan/Alginate (Chi/Alg)/BMP-2 Scaffolds have been shown to support the differentiation of mesenchymal stem cells into bone (8). Neural stem cells proliferate optimally in Chi/Alg hydrogels (9). Studies have shown that the hybrid system of Chi/Alg hydrogel and mesenchymal stem cells could be suitable for wound healing (10). Alginate conduits can be suitable scaffolds for nerve tissue engineering (9). Previous studies have demonstrated that recombinant hydroxyapatite nanoparticles combined with chitosan gel, along with mesenchymal stem cells (MSCs), are effective for bone resorption and regeneration processes (11). SW742 epithelial cells have high viability in magnetic chitosan nanocomposites (12). Nano-

based drug delivery platforms, including nanoparticles, nanosuspensions, nanomicelles, and nanoemulsions, can significantly enhance the delivery of hydrophobic compounds. To mitigate drug-related limitations, natural polymers are considered ideal candidates for nanoparticle fabrication owing to their inherent biodegradability and biocompatibility (13). Today, there is a need to provide optimal biomaterials for applications in tissue engineering. For scaffold synthesis, natural polymers are outstanding due to their biodegradable, non-toxic, and biocompatible properties (14). Chitosan is a linear polysaccharide with repetitive units of D-Glucosamine and N-acetylglucosamine. It is biocompatible; hence, it has been used in biological and biomedical applications. Moreover, chitosan has antibacterial properties due to the presence of amine groups in its chemical structure (1, 2). In this regard, another notable polysaccharide is alginate. Alginate, obtained from brown algae and certain bacteria such as *P. aeruginosa*, is widely used to form gels and films (15). Electrostatic interactions between the chitosan amine group and the alginate carboxyl groups form a polyelectrolyte complex. This complex retains the characteristics of each polymer and creates changes in the swelling tendency (16). Designing effective drug delivery systems has been an attractive area of research in recent years. These systems cannot only improve the pharmacological properties of drugs but also reduce side effects by limiting drug availability to healthy tissues (17). Targeted drug delivery systems as biopolymer/nanomaterial composites have attracted much attention and have improved controlled drug release (17). The drugs loaded in nanoparticles can be incorporated into the scaffold to sustain delivery without an obvious initial burst from the nanoparticles (18). Previous studies indicated that scaffolds and nanoparticles can be used as a long-term drug delivery system (19).

Despite these advances, current scaffold–nanoparticle systems still face critical challenges, including initial burst release, limited control over sustained drug release, and insufficient integration of drug carriers within three-dimensional matrices. To address these limitations, the present study proposes a Chi/Alg porous scaffold incorporating silibinin-loaded chitosan nanoparticles (Chi/Alg/SCNPs) as an integrated drug-delivery platform. In this system, chitosan nanoparticles enhance the encapsulation and solubility of hydrophobic silibinin, while the chitosan/alginate scaffold provides a mechanically robust and highly porous structure that supports cell adhesion and proliferation. Embedding SCNPs within the scaffold matrix is expected to reduce the initial burst release and enable a more sustained and controlled release profile. This combinatorial design may generate a synergistic effect, improving drug bioavailability, modulating release kinetics, and enhancing cytocompatibility and antibacterial performance. Therefore, the Chi/Alg/SCNPs system is anticipated to outperform conventional scaffolds or nanoparticle-based approaches alone, offering a more efficient platform for tissue engineering applications.

Materials and Methods

Chitosan (degree of deacetylation >75%, MW = 190–375 kDa), sodium alginate, sodium tripolyphosphate (TPP), silibinin, calcium chloride (CaCl₂), and glutaraldehyde solution (1% w/v) were obtained from Sigma-Aldrich (USA). Dulbecco's Modified Eagle Medium, fetal bovine serum (FBS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent were purchased from Invitrogen (USA). All other chemicals, including sodium hydroxide (NaOH), acetic acid, and ethanol, were of analytical grade and used without further purification.

The C6 glioma cell line (rat-derived) was obtained from the Pasteur Institute (Tehran, Iran).

Bacterial strains, including *Staphylococcus aureus* (PTCC 1112) and *Escherichia coli* (PTCC 1330), were purchased from Pasteur Institute (Tehran, Iran). All biological experiments were performed under standard sterile laboratory conditions.

Synthesis of Silibinin-Loaded Chitosan Nanoparticles (SCNPs)

Silibinin-loaded chitosan nanoparticles (SCNPs) were synthesized using the ionic gelation method. Chitosan (molecular weight: 190–375 kDa; degree of deacetylation >75%) was dissolved in 1% (v/v) acetic acid to obtain a 0.5% (w/v) solution, and the pH was adjusted to 4.3. Sodium TPP (0.25% w/v) was prepared in distilled water and filtered prior to use. Silibinin (6 mg/mL in DMSO) was added dropwise to the chitosan solution under continuous stirring, followed by sonication for 2 min (20).

Subsequently, TPP solution was added dropwise to the chitosan solution at a chitosan: TPP mass ratio of 10:4 under constant stirring (500 rpm) for 60 min to induce nanoparticle formation via ionic crosslinking. This ratio was selected based on previous studies demonstrating optimal nanoparticle size, stability, and encapsulation efficiency using similar conditions (21).

The nanoparticle suspension was centrifuged at 15,000 rpm for 30 min, washed twice with distilled water, and lyophilized. All experiments were performed in triplicate (n = 3) (22).

Drug Loading Efficiency

A suspension of SCNPs synthesized at 15000 rpm was centrifuged for 30 minutes to separate the untrapped drug. The supernatant was collected, and 4 mL of ethanol was added. The resultant solution was analyzed using a Shimadzu UV–Vis 1650 PC spectrophotometer (Kyoto, Japan) at 287 nm. The value of encapsulation efficiency (EE) was calculated based on the following formula (23):

EE (%) = [(Amount of silibinin added – Amount of silibinin in the supernatant) / Amount of silibinin added] × 100.

All measurements were performed in triplicate (n=3).

Design of Silibinin-Loaded Chitosan Nanoparticles (SCNPs)-Incorporated into the Scaffold (Chi/Alg- SCNPs)

The Chi/Alg scaffold was fabricated using a previously reported method with slight modifications. Chitosan solution was prepared by dissolving 4.8 g of chitosan in 80 mL of 1 N acetic acid. A total of 4.8 g of sodium alginate in 120 mL 1 N NaOH was dissolved. The SCNPs (1 mg/mL) were added to the alginate solution with stirring. Then the chitosan solution was poured into the previous solution. The resultant solution was sonicated and stirred to obtain a homogeneous dispersion of 4.8% w/v (2.4% chitosan, 2.4% alginate). By adding 2 N acetic acid solution, the pH of the solution was set to 7.4. The solution was then placed into a 24-well plate, maintained at 20°C for 24 h, and then lyophilized in a freeze dryer. A total of 0.2 M CaCl₂ solution was used to crosslink the scaffolds. Pure chitosan scaffolds were also prepared for comparison (24).

Morphological Analysis

Scaffolds were freeze-dried for scanning electron microscopy (SEM). Then, thin sections of scaffolds were prepared and gold-coated. SEM images were taken using a LEO 440i scanning electron microscope (TESCAN Company, Brno, Czech Republic). The liquid displacement method was used to determine the total porosity of samples. To calculate porosity, initially the dry weight of the samples and the volume of ethanol were measured. Then, the samples were immersed in dehydrated alcohol for 48 h, and their weights were measured again. Finally, the porosity of the samples was estimated based on the following formula: where V₁ = the initial known weight of the samples, V₂ = the sum of

the weights of ethanol and the submerged samples, and V₃ = the weight of ethanol after the removal of the samples. Three parallel sets were analyzed for every sample, and the mean porosity for each sample was calculated (25).

$$\text{Porosity\%} = (V_2 - V_1 - V_3) / (V_2 - V_3) \times 100$$

Structural Analysis

Fourier transform infrared (FTIR) spectroscopy was performed using a Bruker-Tensor 320 spectrometer. X-ray diffraction patterns of scaffolds were recorded using a Siemens D-5000 X-ray diffractometer (Germany) with Cu K radiation at 45 kV and a scan range of 2θ = 2° to 60°. The thermal stability of the Chi-Alg and Chi-Alg-SCNPs was analyzed by using Mettler Toledo simultaneous thermogravimetric analysis (TGA). The samples were heated from 25°C to 800°C at a heating rate of 10°C /min under a nitrogen atmosphere (26).

Swelling and Degradation Studies

The swelling ratio was determined by immersing scaffolds, weighed equally, in 1× phosphate-buffered saline (PBS) solution for 12 days (1, 3, 6, 9, 12 days) at pH = 7.4 and 37°C. The swelling ratio was obtained by the following formula, where W_w was the wet weight (after swelling in PBS), and W_D was the dry weight of the scaffolds (27).

$$\text{Swelling ratio (\%)} = [(W_w - W_d) / W_d] \times 100$$

Scaffolds of equal weight were used to estimate the rate of biodegradation. Initial weight was recorded as W_i, followed by immersion in sterile Falcon tubes containing 50 mL PBS (pH: 7.4). Scaffolds were incubated at 37°C at different time intervals (1, 3, 6, 9, 12 days). After each incubation period, the scaffolds were rinsed with deionized water to remove soluble inorganic salts and then dried in an oven at 60°C for 24 h. The dry weights of the scaffolds were noted as (W_t). The biodegradation percentage was calculated by using the following formula (28).

$$\text{Biodegradation (\%)} = [(W_i - W_t) / W_i] \times 100$$

Mechanical Properties

Mechanical properties (tensile stress) of scaffolds were evaluated using a universal testing machine (Instron 5566, USA). The samples were prepared with dimensions of 5 cm × 1 cm × 1 cm. The loading rate of 5 mm/min was set at room temperature.

Drug Release Assay

The profiles of silibinin release from SCNPs were obtained using the dialysis bag membrane method at different pH (5.4 and 7.4) at 37°C. Briefly, SCNPs (59 mg in 3 mL of PBS) were added to a dialysis bag. The dialysis bag was immersed in 40 mL of PBS, then stirred at 100 rpm at 37°C. Samples (containing 4 mL of the released silibinin) were removed at predetermined time intervals, and the same volume was replaced with fresh PBS. Also, release profiles of silibinin from Chi-Alg-SCNPs, Chi-SCNPs, and Alg-SCNPs scaffolds were obtained. Samples with dimensions of 3 × 3 cm were dispersed in 20 mL PBS solution, pH 7.4 and 5.4, at 37°C. At predetermined time intervals, the supernatant was pipetted out and replaced with equivalent volumes of fresh phosphate buffer solution. The amount of released silibinin was quantified by 6715 UV/Vis spectrophotometry (JENWAY) at 287 nm using the silibinin standard calibration curve. The percentage of the released silibinin was calculated using the following equation (29):

$$\text{Drug release (\%)} = [(\text{Amount of silibinin released}) / (\text{Initial amount of silibinin loaded})] \times 100$$

Antibacterial Activity

The colony counting method was used to investigate the antibacterial properties of SCNPs, Chi-Alg, Chi-Alg-SCNPs, Chi-SCNPs, and Alg-SCNPs scaffolds using Gram-negative bacteria (*Escherichia coli*; *E. coli*) against Gram-positive bacteria (*Bacillus cereus*, *B. cereus*) as model

organisms. Monocolonies of *E. coli* and *S. aureus* were cultured in liquid Luria-Bertani (LB) culture medium and then incubated at 37°C for 12 h. 10⁶–10⁷ CFU/ ml was used to dilute the bacteria with PBS. Then, 1 mL of *E. coli* and *S. aureus* bacterial suspension with 1 µg of the samples was mixed in the dark for 30 min. Afterward, 150 µL of the suspension was spread on LB agar plates. After 12 h of incubation at 37°C, colonies were counted to evaluate the viability of *E. coli* and *S. aureus* cells in the samples (30).

Cellular Studies

Rat bone marrow-derived mesenchymal stem cells (Rat BMSCs) were employed to estimate the cell viability on scaffolds. Rat BMSCs cultures were prepared according to the procedure described previously with only minor modifications. Male Wistar rats of 4-6 weeks were purchased from the Iranian Institute of Pasteur. Before the study began, all animal experiments were approved by the Ethical Committee of Lorestan University of Medical Sciences (LUMS.REC.1396.270). The rats were sacrificed with ketamine-xylazine (K, 100 mg/kg; X, 10 mg/kg). The femurs were removed and dissected without adherent soft tissue. An insulin syringe containing medium was inserted into the bone marrow cavity. The contents of the syringe (medium and the bone marrow) were discharged under pressure into a 25 cm² plastic culture flask containing medium with 10% FBS and 1% penicillin/streptomycin. The plastic culture flask was incubated at 37 °C under 5% CO₂ and 95% air. The adherent cells were allowed to reach ~90% confluence.

The MTT assay was used to evaluate cell viability by monitoring mitochondrial function. Cells were used in their second to third passage. Different scaffold groups (Chi/Alg, Chi- SCNPs, Chi/Alg-SCNPs and Chi) were cut into small pieces and exposed to UV radiation for 1.5 h and then placed in 96-well plates, and cells were seeded onto the scaffolds with a density of 7×10⁴ cells and incubated for 24 h. Then, the culture medium was

removed, and fresh medium containing 100 μ L of MTT was added to each well for 4 h in the dark. Then, the MTT solution was removed and replaced with 100 μ L DMSO in each well. After 20 min, the absorbance of the solution was read by an ELISA reader at 490nm. In addition, field emission SEM observations were used to evaluate cell interactions.

Statistical Analysis

All experiments were performed in triplicate ($n = 3$), and data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was conducted using SPSS software (version 22).

One-way analysis of variance followed by Tukey's post hoc test was used for multiple comparisons, while an independent t-test was applied for two-group comparisons. A P value < 0.05 was considered statistically significant.

Results and Discussion

Morphological Results

The SEM analysis was performed to determine the particle size of SCNPs and to examine the surface morphology, architecture, pore size, and pore structure of Chi/Alg, Chi/Alg/ SCNPs, Chi, Chi/ SCNPs Alg, Alg/SCNPs scaffolds (Fig. 1).

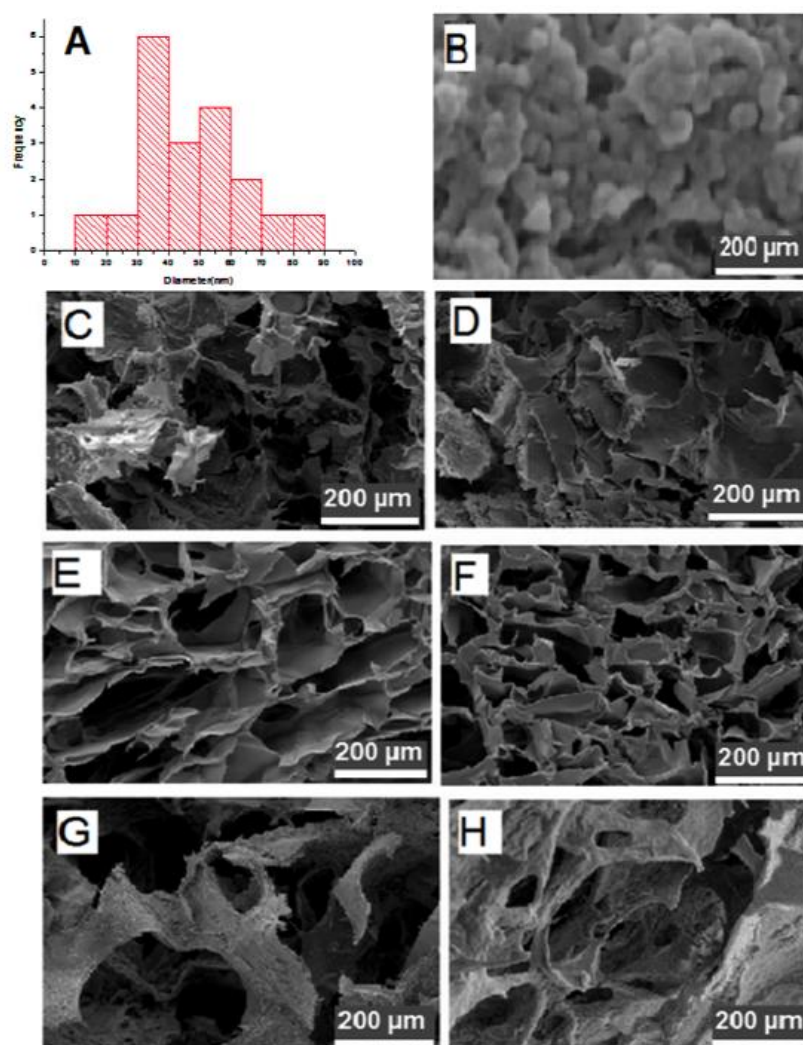


Fig. 1. Particle Size Distribution of Silibinin-Loaded Chitosan Nanoparticles (SCNPs).

(A). Microstructures of SCNPs (B), Chi/Alg (C) Chi/Alg/ SCNPs(D), Chi, (E) Chi/ SCNPs (F) Alg(G) and Alg/SCNPs (H)scaffolds were analyzed by SEM. Scale bar 200 μ m.

ImageJ analysis of SEM micrographs indicated that the average particle size was 44.80 nm (Fig. 1 A, B). Figs. 1C, 1E, and 1G showed images of Chi/Alg, Chi, and Alg scaffolds, respectively. This scaffold is suitable for nanoparticle incorporation due to its uniform, porous structure. The pores were uniformly distributed with sizes ranging from 150–200 μm . The porosity results

showed that the scaffolds had about 90% porosity (Fig. 2). A comparative decrease in porosity was observed in scaffolds containing nanoparticles. Cell migration into the scaffold is an important index of high porosity. High porosity can be an important factor for tissue engineering. This feature of porosity can indicate the scaffold's suitability for cell attachment and migration (31).

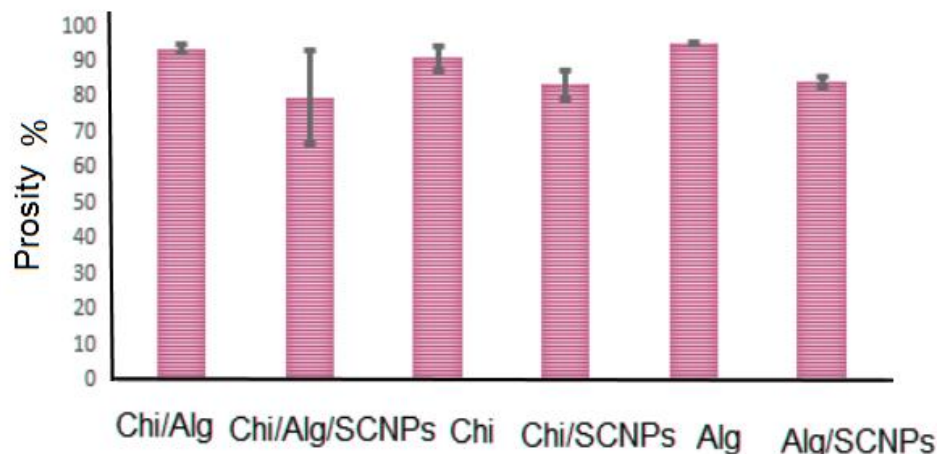


Fig. 2. Mean Porosity of Different Scaffolds of Alginate and Chitosan (Chi/Alg), Chi/Alg/SCNPs, Alg, Alg / SCNPs, Chi, Chi/ SCNPs.

Structural Analysis

The chemical interaction between silibinin, Chi, Alg, and SCNPs was analyzed by FTIR spectroscopy (Fig. 3A). Silibinin (Fig.3a) showed characteristic peaks at 3418 cm^{-1} (O–H stretching), 1609 cm^{-1} (C=O stretching), 1460 cm^{-1} (aromatic C–C stretching vibrations), and 1261 cm^{-1} (C–O–C stretching). FT-IR analysis of Chi (Fig.3c) showed the characteristic peaks at 3448 cm^{-1} (O–H stretching), 1610 cm^{-1} (C=O stretching), and 1094 cm^{-1} (C–O stretching). In the FT-IR spectrum of silibinin loaded with nanoparticles (Fig. 3b), characteristic absorptions of silibinin and Chi were observed at 3439 cm^{-1} (O–H stretching), 1558 cm^{-1} (amide I stretching), and 1398 cm^{-1} (amide III stretching). In the IR spectrum of Chi- SCNPs (Fig. 3d), characteristic absorptions at 3448 cm^{-1} (OH stretching) and 1094 cm^{-1} (N–H stretching) were shifted to 3474 and 1402 cm^{-1} , respectively, which represents the

interaction between amine groups in chitosan and OH groups in silibinin. FT-IR analysis of alginate (Fig.3e) showed the characteristic peaks at 1643 cm^{-1} , 1402 cm^{-1} , and 1130 cm^{-1} that are attributed to stretching vibrations of asymmetric and symmetric bands of carboxylate anions, and antisymmetric stretch C–O–C, respectively. In the FT-IR analysis of Alg-SCNPs (Fig.3f), peaks at 1634 cm^{-1} and 1128 cm^{-1} are attributed to stretching vibrations of carboxylate anions and C–N stretching vibrations, respectively. The FT-IR spectra of Chi-Alg-SCNPs showed characteristic peaks of Chi, Alg, and SCNPs and a band shift compared with those of the nanocarrier and pure drug. As presented in Fig. 3 (c), the peaks at 1585 cm^{-1} and 1415 cm^{-1} in Chi-Alg were altered to 1622 cm^{-1} and 1119 cm^{-1} in Chi-Alg- SCNPs. These changes demonstrated that the ionic interaction formed a chitosan-alginate scaffold

between the negatively charged carboxyl group ($-\text{COO}^-$) of alginate and the positively charged amino group (NH_3^+) of chitosan X-ray diffraction patterns of silibinin (Fig. 3B (C)) showed sharp peaks at 13.66° , 14.61° , 16.11° , 17.41° , 19.69° , 20.75° , 22.38° , indicating the crystalline nature of silibinin. X-ray diffractogram of Alg (Fig. 3B (H)) showed a sharp peak at 27.14° while no such crystalline diffraction peaks were observed in the diffractograms of the scaffolds. This indicates that the crystalline form of silibinin and Alg has disappeared, and that the drugs were successfully loaded into the scaffolds. The decreased

crystallinity of the Chi-Alg scaffold also implied that ionic interactions between chitosan and alginate lead to their best compatibility, consistent with the FTIR results. The thermal stability of the Chi/Alg and Chi/Alg/ SCNPs was conducted by TGA between 20 and 800°C under a nitrogen atmosphere. Thermal gravimetric analysis of Chi/Alg (1a) and Chi/Alg/ SCNPs (1b) showed that both of them had a temperature variation of 200°C and 500°C (Fig. 4). In fact, the incorporation of nanoparticles into the Chi/Alg scaffold did not change the thermal stability.

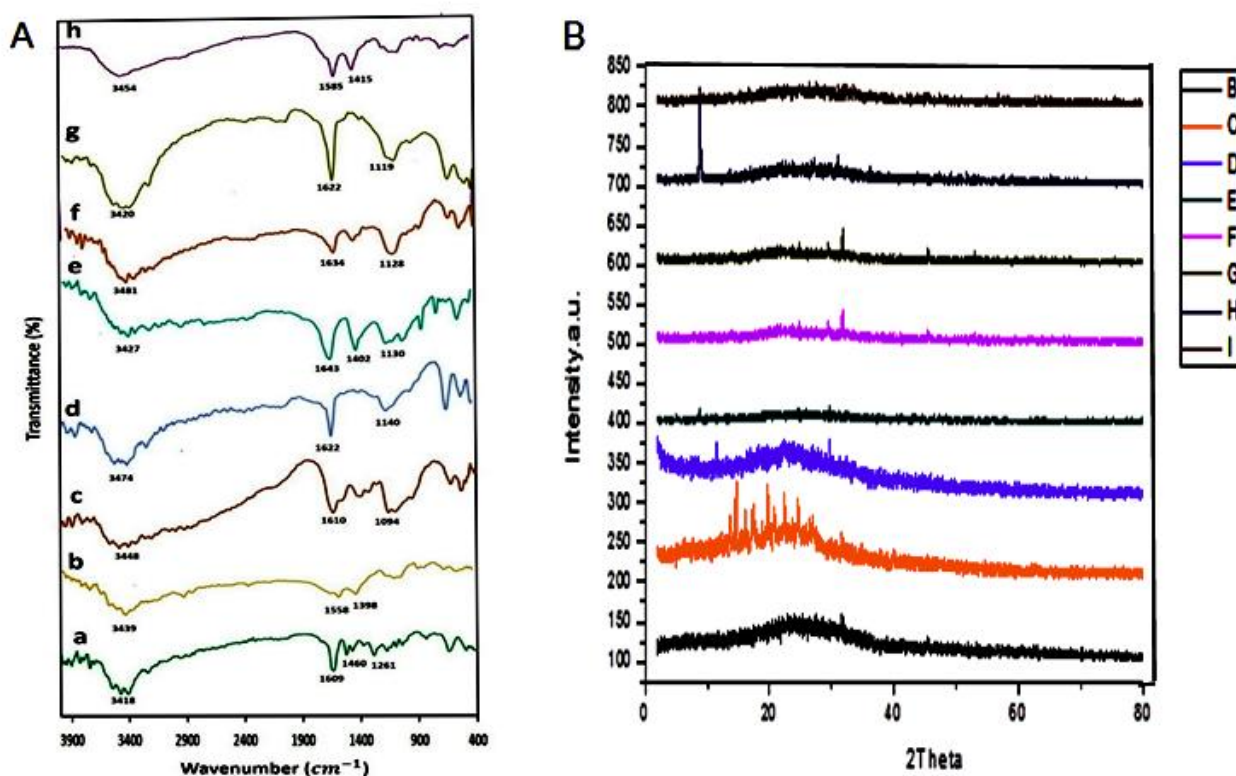


Fig.3. A) FTIR Spectra of (a) Silibinin, (b) Silibinin-Loaded Chitosan Nanoparticles (SCNPs), (c) Chi, (d) Chi/ SCNPs, (e) Alg, (f) Alg/ SCNPs, (g) Chi/Alg SCNPs and (h) Chi/Alg. B) X-ray diffraction pattern of (B) Chi, (C) Silibinin (D) SCNPs (E) Chi/Alg (F) Chi/Alg/SCNPs (G) Chi/SCNPs (H) Alg and (I) Alg/SCNPs.

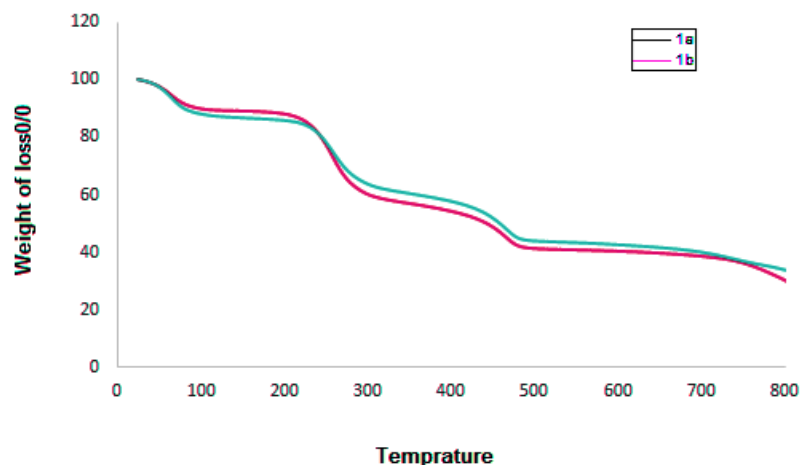


Fig.4. Thermogravimetric Analysis (TGA) Thermograms of Alginate and Chitosan (Chi/Alg) (1a) and Chi/Alg /SCNPs (1b).

Swelling and Degradation Test

The swelling behavior of Chi/Alg, Chi/Alg/SCNPs, Chi, and Chi/SCNPs scaffolds was evaluated by floating these scaffolds in 1×PBS solution for 1, 3, 6, 9 and 12 days. Since alginate was destroyed very quickly, results on swelling behavior, biodegradation, and cytotoxicity were excluded. The results showed

that impregnation of SCNPs in Chi/Alg and Chi/Alg scaffolds decreased the swelling ratio at 3, 6, 9, and 12 days. These changes were significant among these scaffolds ($P < 0.001$). However, SCNPs impregnation did not significantly affect the swelling ratio after 1 day of incubation (Fig. 5).

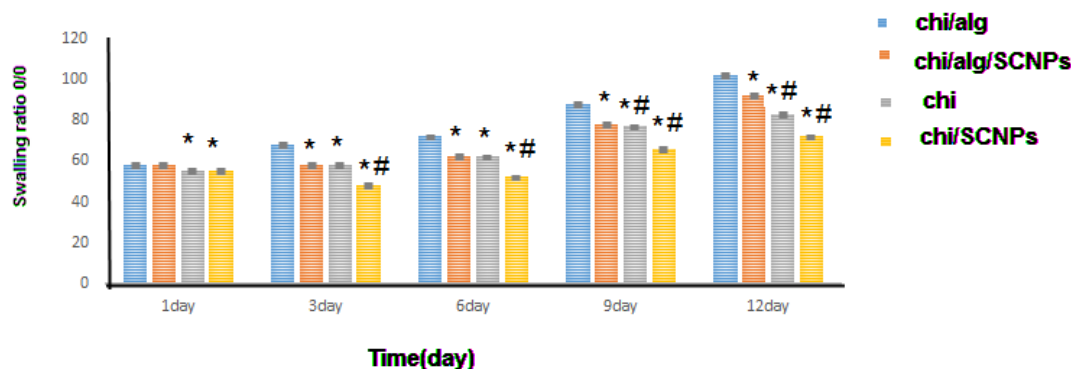


Fig. 5. Swelling Behavior of Alginate and Chitosan (Chi/Alg), Chi/Alg /SCNPs, Chi, Chi/ SCNPs in PBS at 37°C for 1,3,6,9,12 days. Swelling ratio was found to be decreased by addition of SCNPs. * $P < 0.001$ versus the Chi/Alg group and # $P < 0.001$ versus the Chi/Alg /SCNPs group.

By absorbing 200–300 times more water than its own weight, alginate facilitates nutrient transport and cellular infiltration. Also, due to the electrostatic interaction of alginate with chitosan, few free functional groups remained to form

hydrogen bonding with water in Chi/Alg scaffolds. Increased swelling led to enhanced surface area and pore size, which can result in better cellular attachment, infiltration, and nutrient availability (32). Though the Chi/Alg/

SCNPs scaffolds exhibited decreased swelling, the claim of increased surface area is not supported by experimental evidence. To analyze the biodegradation rate, scaffolds were incubated in PBS solution for 1, 3, 6, 9 and 12 days. A significant increase in the degradation of Chi/Alg/ SCNPs and chi/ SCNPs scaffolds was obtained compared to that of Chi/Alg and chi scaffolds at all incubation time periods (Fig. 6). Compared to chi/Alg scaffolds, the enhanced degradation of Chi/Alg /SCNPs could be due to crosslinking of alginate with calcium ions and chitosan.

The degradation behavior of the scaffolds can be attributed to the interplay between crosslinking density and structural composition. Generally, increased crosslinking, such as ionic interactions between alginate and Ca^{2+} , is expected to enhance structural stability and reduce degradation. However, in the Chi/Alg/SCNPs scaffolds, the observed increase in degradation compared to Chi/Alg scaffolds may be explained by the incorporation of SCNPs, which can disrupt the uniform polymer network and create additional porosity (Fig. 6).

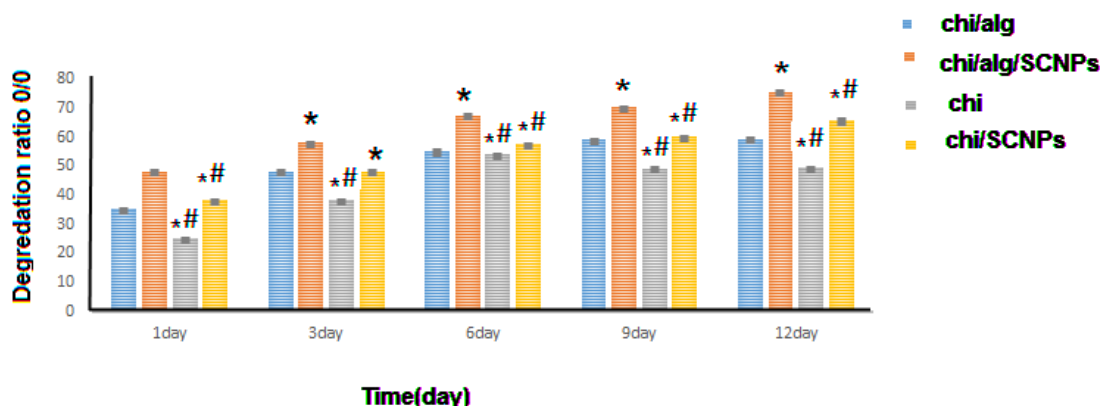


Fig. 6. Biodegradation Rate of Alginate and Chitosan (Chi/Alg), Chi/Alg /SCNPs, Chi, Chi/ SCNPs in PBS at 37°C for 1,3,6,9,12 days. * $P < 0.001$ versus the Chi/Alg group and # $P < 0.001$ versus the Chi/Alg /SCNPs group.

Moreover, the release of SCNPs from the scaffold matrix likely contributes to accelerated mass loss, as their diffusion leaves behind voids that weaken the structural integrity of the scaffold. This effect may explain the higher degradation rate of Chi/Alg/SCNPs compared to Chi/SCNPs. Similar findings have been reported in previous studies, where incorporating chitosan-based nanoparticles into alginate-based 3D porous scaffolds resulted in enhanced biodegradation due to increased porosity and reduced matrix cohesion (33).

Drug Release Assay

The loading efficiency of Silibinin in SCNPs was 87%. The ability of the constructed scaffolds to deliver Silibinin to target sites was evaluated. The release of silibinin from SCNPs, Chi /Alg /SCNPs, Chi/SCNPs, Alg/SCNPs was determined up to 120 h at pH = 7.4 (Fig. 7). After 120 h, approximately 50% of the drug was released from SCNPs at pH = 7.4. The percentage of silibinin released from Alg/SCNPs, Chi/Alg–SCNPs, and Chi/SCNPs, (at pH = 7.4) was approximately 40%, 31 %, and 23% at 120 h, respectively. Due to high swelling, alginate scaffolds release drugs faster than chitosan ones. It is suggested that the scaffold should act as a

barrier to Silibinin release, preventing the initial burst release and allowing sustained release due to scaffold degradation.

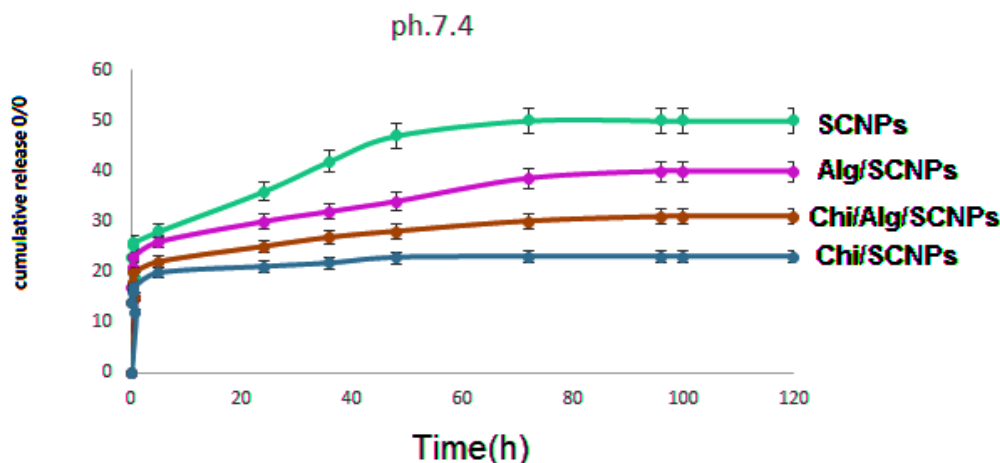


Fig. 7. Release Profile of Silibinin-Loaded Chitosan Nanoparticles (SCNPs), Alginate and Chitosan (Chi /Alg) / SCNPs, Chi/ SCNPs, Alg/ SCNPs at pH 7.4.

Mechanical Testing

The stress–strain curve obtained from the tensile test showed that the Young's modulus was increased by the addition of chitosan in the composite (Chi/Alg), and that the highest modulus belonged to the composite sample containing nanoparticles. The Chi/Alg scaffolds containing nanoparticles demonstrated tensile stress and Young's modulus of 2703.6 kPa and 30.7 MPa, respectively, while the Chi/Alg scaffolds demonstrated tensile stress and Young's modulus of 683.7kPa and 12.8 MPa, respectively. The addition of SCNPs into Chi/Alg scaffolds increased Young's modulus and tensile stress and decreased the elongation.

Antibacterial Activity

Antibacterial activity was assayed by the colony count method (Fig. 8). The viability of *E. coli* cells was significantly reduced ($P < 0.001$) by $51.62 \pm 6.83\%$ and $52.71 \pm 6.83\%$, $41.52 \pm 6.83\%$, $51.26 \pm 6.83\%$, $51.26 \pm 3.41\%$, $69.68 \pm 3.41\%$, $70.40 \pm 3.41\%$, $96.39 \pm 3.41\%$, $84.84 \pm$

3.41% , $97.47 \pm 3.41\%$, and the viability of *B. cereus* cells was significantly decreased ($P < 0.001$) by $37.82 \pm 6.83\%$, $30.25 \pm 6.83\%$, $22.27 \pm 6.83\%$, $40.76 \pm 6.83\%$, $57.56 \pm 6.83\%$, $84.03 \pm 3.41\%$, $94.12 \pm 3.41\%$, $81.51 \pm 3.41\%$, and $95.38 \pm 3.41\%$ after being incubated with silibinin, Alg, Chi, CNPs, SCNPs, Chi/Alg, Alg/SCNPs, Chi/SCNPs, Chi/Alg/SCNPs aqueous suspensions, respectively. SCNPs, Chi/SCNPs, Alg/SCNPs, Chi/Alg/SCNPs showed more antibacterial activity against Gram-negative than Gram-positive bacteria. Antibacterial activity of synthesized SCNPs was higher against the Gram-negative bacteria than the Gram-positive. This result was expected because silibinin has shown antibacterial activity against Gram-negative (*E. coli*) (34). Based on the results, the antibacterial property of the scaffolds increased with SCNPs impregnation, and the Chi/Alg/SCNPs scaffold had stronger antibacterial activity. On the other hand, the antibacterial properties of the tested samples against the Gram-negative bacteria were higher than against Gram-positive bacteria,

which may be due to the more negative surface charge of Gram-negative bacteria (*E. coli*) than that of the Gram-positive bacterium (*B. cereus*) (35). It is probable that positive-charge nanoparticles (SCNPs) had a higher affinity for Gram-negative bacteria than Gram-positive bacteria (36), and as a result, antibacterial activity

increased. The higher antibacterial capacity of the Chi/Alg/SCNPs scaffold is likely related to its higher release capacity during the first minutes. Moreover, our results suggest that Alg/SCNPs have strong antibacterial activity against *E. coli* bacteria.

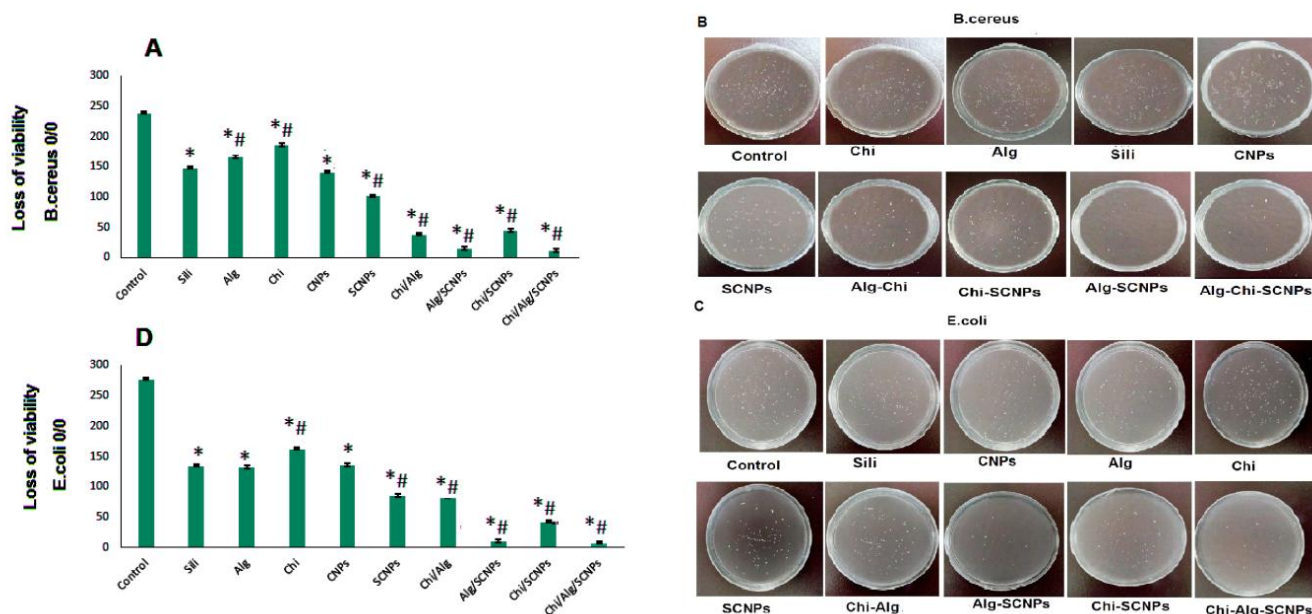


Fig. 8. Antibacterial Activity of Sili, Alg, Chi, SCNPs, CNPs, Alginate and Chitosan (Chi/Alg), Alg- SCNPs, Chi-SCNPs, Chi/Alg- SCNPs against *B. cereus* (A) and *E. coli* (D). (B & C) Representative photographs of *E. coli* and *B. cereus* colonies grown on agar plates after exposure to samples.

* $P < 0.001$ versus the control group and # $P < 0.001$ versus the sili (Silibinin) group.

Cell Viability

MTT assay was used to evaluate the viability of the rat BMSC cell line on Chi/Alg SCNPs, Chi/SCNPs, Chi and Chi/Alg. The survival of Rat BMSCs in Chi/Alg SCNPs and Chi/SCNPs was significantly higher than that in other scaffolds ($P < 0.001$, Fig. 9E), which indicated that the

impregnation of SCNPs in the Chi/Alg scaffolds and Chi scaffolds was tolerated by Rat BMSCs. Fig. 9 (A-D) shows the morphology of the Rat BMSCs cell line on the scaffolds with SEM analysis. The images show improved attachment and growth of the Rat BMSC cell line on the Chi/Alg/SCNPs and Chi/SCNPs scaffolds.

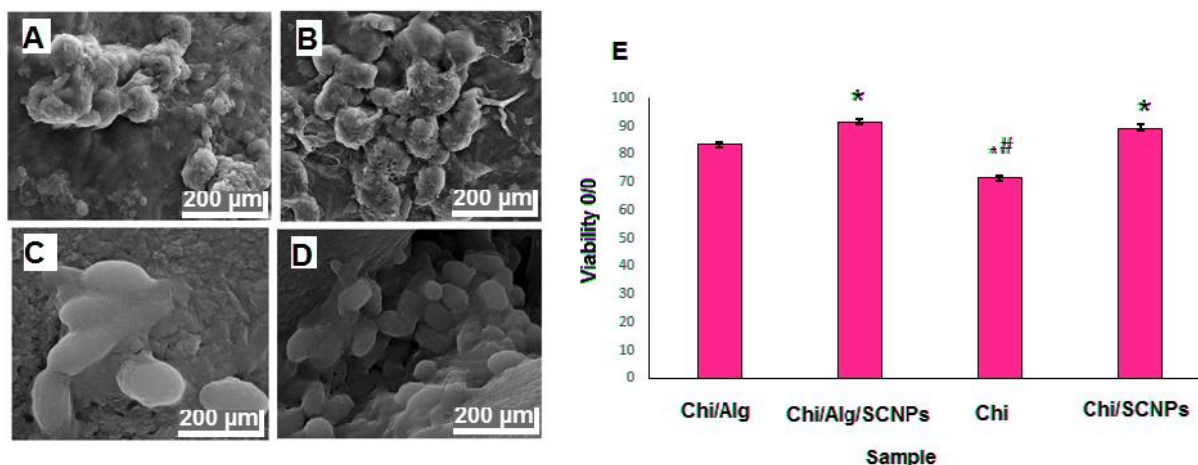


Fig.9. A) Scanning Electron Microscopy (SEM) Images of Rat Bone Marrow-Derived Mesenchymal Stem Cell (BMSC) Adhesion on Alginate and Chitosan (Chi/Alg) (A) Chi/Alg /SCNPs, (B) Chi (C) Chi/ SCNPs(D). (E) 3-(4,5- dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide (MTT) assay of Rat BMSCs cell viability on Chi/Alg, Chi/Alg /SCNPs, Chi and Chi/ SCNPs. * $P < 0.001$ versus the Chi/Alg group and # $P < 0.001$ versus the Chi/Alg /SCNPs group.

Conclusion

In this study, a Chi/Alg scaffold incorporated with silibinin-loaded chitosan nanoparticles (Chi/Alg/SCNPs) was successfully developed and characterized. The results demonstrated that incorporating SCNPs influenced the scaffold's physicochemical properties, degradation behavior, and drug-release profile. The system showed improved controlled release characteristics and suitable cytocompatibility under in vitro conditions. However, these findings are limited to in vitro evaluations, and further studies are required to confirm the performance of this system in more complex biological environments. Future research should focus on in vivo validation, optimization of scaffold composition, and a deeper investigation of the underlying mechanisms governing drug release and tissue interactions. Overall, the Chi/Alg/SCNPs system may represent a promising platform for controlled drug delivery in tissue engineering applications, although additional studies are necessary to fully establish its clinical potential.

Acknowledgements

The authors gratefully acknowledge support from Lorestan University of Medical Sciences, Lorestan, Iran (grant number A-10-1416-3).

Author Contributions

All authors contributed to the study conception and design. Maryam Alipour, Faraz Souri, and Mojtaba Khaksarian performed material preparation, data collection, and analysis. Maryam Alipour and Mojtaba Khaksarian wrote the first draft of the manuscript, and all authors reviewed and commented on previous versions. All authors approved the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

All authors have read and agreed to the published version of the manuscript.

Funding Statement

The authors gratefully acknowledge support from Lorestan University of Medical Sciences, Lorestan, Iran (grant number A-10-1416-3).

Conflict of Interest

The authors declared no conflicts of interest.

Ethics Statement

Before the study began, all animal experiments were approved by the Ethical Committee of Lorestan University of Medical Sciences (LUMS.REC.1396.270).

Data Availability Statement

The datasets used and/or analyzed during the current study will be available from the corresponding author on reasonable request.

Artificial Intelligence Statement

During manuscript preparation, the authors used ChatGPT to edit certain sentences. The authors bear full responsibility for the content.

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