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Development of 3D Printed Scaffolds Containing Decellularized Plants and Investigation of Their Basic Cell Interactions

Hücresizleştirilmiş Bitki İçeren 3B Baskılı Doku İskelelerinin Hazırlanması ve Temel Hücre Etkileşimlerinin İncelenmesi

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ABSTRACT

The decellularization process fundamentally removes the cellular content of the tissue (nuclear material and other nucleic acid components) without disrupting the structural integrity of the tissue. It is an effective approach, especially for obtaining three-dimensional (3D) biomaterials composed of the extracellular matrix (ECM), which provides tissue biomechanical support. In the literature, studies have shown that after the decellularization process, animal-derived decellularized tissues have been combined with various biopolymers to prepare composite scaffolds using different techniques. In recent years, due to their structural features, decellularization studies of plant-derived tissues have also gained prominence alongside animal tissues. In this study, succulent plants were chosen as the plant tissue, and the purpose was to prepare hybrid scaffolds by combining decellularized succulent tissues with alginate structures and to investigate the fundamental cell-material interactions using mesenchymal stem cells. Succulent plant leaves were decellularized using a solution containing Triton X-100 and SDS. The water-retaining parts were separated from other tissues, lyophilized, and turned into a powder. This approach was employed to preserve biomolecules with water-retaining capacity in powdered form. To determine the efficiency of the decellularization process, the quantities of DNA and proteins were assessed and compared. Due to their high water-absorbing capacity, the succulent plants' water-retaining structures were combined with alginate biopolymer at various viscosity levels to prepare an ink suitable for 3D printing. After printing, the resulting scaffolds' degradation and swelling behavior, chemical composition, structural characterization, and thermal properties were examined. In the final phase, a fundamental investigation was carried out on cell-material interactions using L929 mouse fibroblast cells and human mesenchymal stem cells on 3D-printed scaffolds. The interactions within the prepared hybrid scaffolds were analyzed through basic cytotoxicity tests.

Key Words
Succulent plant, plant decellularization, biomaterials.

ÖZ

Hücresizleştirme işlemi, dokularda biyomekanik destek sağlayan ve hücre dışı matristen (ECM) oluşan 3B biyomalzemelerin elde edilmesinde kullanılmak üzere, dokudaki hücresel içeriğin dokunun yapısal bütünlüğünü bozmadan uzaklaştırılması işlemidir. Literatürde yapılan çalışmalar, hücresizleştirilmiş hayvan kaynaklı dokuların, çeşitli biyopolimerlerle farklı teknikler kullanılarak birleştirilerek kompozit doku iskeleleri hazırlandığını ortaya koymuştur. Son yıllarda yapısal özellikleri nedeniyle hayvan dokularının yanı sıra bitki kaynaklı dokuların da hücresizleştirme çalışmaları ön plana çıkmıştır. Bu çalışmada, hücresizleştirilmiş sukulent dokuları aljinat ile birleştirilerek hibrit doku iskeleleri hazırlanması ve mezenkimal kök hücreleri kullanılarak temel hücre- malzeme etkileşimlerini araştırmaktır. Kullanılan metoda göre, etli bitki yaprakları hücresizleştirilip su tutan kısımlar diğer dokulardan ayrıldı, liyofilize edildi ve toz haline getirildi. Bu yaklaşım, su tutma kapasitesine sahip biyomolekülleri toz halinde korumak için kullanıldı. Hücresizleştirme sürecinin verimliliğini belirlemek için DNA ve protein miktarları değerlendirilip karşılaştırıldı. Yüksek su tutma kapasiteleri nedeniyle etli bitkilerin su tutan yapıları, 3B baskıya uygun bir mürekkep hazırlamak için çeşitli konsantrasyonlarda aljinat biyopolimeri ile birleştirildi. Baskıdan sonra, elde edilen iskelelerin bozunma ve şişme davranışı, kimyasal bileşimi, yapısal karakterizasyonu ve termal özellikleri incelendi. Son aşamada, 3B yazdırılmış iskeleler üzerinde L929 fare fibroblast hücreleri ve insan mezenkimal kök hücreleri kullanılarak hücre-malzeme etkileşimi üzerine temel bir çalışma yürütüldü. Hazırlanan hibrit iskelelerinde hücre- malzeme etkileşimi temel sitotoksisite testleri kullanılarak incelenmiştir.

Anahtar Kelimeler
Sukulent bitkisi, bitki hücresizleştirme işlemi, biyomalzemeler.

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INTRODUCTION

Tissue engineering, a multidisciplinary field, aims to repair or reconstruct native tissues within living organisms. The aim is to explore the critical role of the extracellular matrix (ECM) in governing cellular function and maintaining tissue integrity. Natural polymers such as collagen, gelatin, chitosan, and hyaluronic acid and synthetic polymers such as polylactic acid, polyglycolic acid, and polyurethane serve as source materials for scaffolds and various processing techniques, such as solvent evaporation, direct molding, and 3D printing, are used in the manufacture of these scaffolds [1, 2].

The decellularization technique is an effective method employed for acquiring three-dimensional biomaterials from tissues, characterized by the extracellular matrix (ECM) that provides biomechanical support. This technique aims to remove cellular content from the tissue without causing harm to its overall integrity [3, 4]. The decellularization method used to obtain 3D biomaterials consisting of an extracellular matrix (ECM) that provides biomechanical support begins with the disruption of the cell membrane using physical procedures such as sonication and shaking or ionic solutions. Then, enzymes such as trypsin and nucleases are used to separate the cellular components from the ECM. Then, detergents such as SDS and Triton dissolve the cytoplasmic and nuclear cellular components. Finally, these steps are performed with a combined technique to eliminate the remaining cellular debris from the tissue. Despite the abundance of scaffolds obtained by various techniques, a universally accepted biomaterial for optimal tissue engineering remains elusive. Recent research has focused on natural sources that can mimic ECM. Decellularization, an important technique, is crucial in preserving tissue-specific ECM. The first studies in this field were performed on animal tissues, including the heart, liver, small intestine, and kidney [5]. Additionally, decellularized animal tissues can be used in their entirety or pulverized for use as additives in the production of hybrid and composite biomaterials. In particular, regardless of whether organs are used as whole or in powder form, the primary goal is to preserve the tissue-specific ECM, which contains important biochemical cues for cellular recognition, thereby facilitating cell proliferation and dissemination [6, 7].

The decellularization process can be applied to both animal and plant tissues, although studies often predominantly emphasize the decellularization of animal tissues. The utilization of plant and animal tissues as biomaterials presents a promising biomedical approach, particularly in the field of tissue engineering. This strategy relies on leveraging similarities between the distinct structures of two crucial realms. It encompasses the decellularization and reseeding of plant tissue. In addition to animal tissues, decellularized plant tissues hold significant potential for mimicking both plant and animal vascular network structures. Pioneering examples in this regard have recently emerged in the literature [8].

In recent years, the potential of plant tissues as decellularized biomaterials has been explored. Pioneering studies by Pelling et al. and Gaudette et al. provided the first examples of plant decellularization by demonstrating the functionality of various plant tissues, such as bamboo, vanilla, and orchid, in both in vitro and in vivo experiments [9, 10, 11]. This process has been extensively examined in both in vivo and in vitro studies. Additionally, there are several examples in the literature that the researchers tried to explore the potential of the use of plant tissues as tissue engineering scaffolds [12, 13, 14].

There are approximately 1300 species of succulent plants belonging to the Crassulaceae family. The succulent plant of the genus Echeveria was used in the study. These plants have high water retention capacity with their specialized storage parenchyma tissues (hydrenchyma). The parenchyma tissue in plants can be considered as the main transmission line responsible for photosynthesis, transport and storage in the plant. Depending on the type of succulent and the amount of water stored in the parenchyma storage parenchyma tissue can reach up to 95% with the contribution of specialized cells [15]. In the basic strategy here, cells secrete high amounts of water-absorbing apoplastic polysaccharides from their elastic cell wall to the extracellular region, and a mucin (glue)-like structure is formed by the binding of water in the environment by these polysaccharides and water is retained in the structure [16].

In this research paper, succulent plant leaves were decellularized using established combined techniques from the literature. Following this process, specialized parenchymal tissues were isolated from other tissues, lyophilized, and transformed into a powdered form. This approach was aimed to preserve the structure of

biomolecules, enhance their water-holding capacity, and positively influence cellular behavior. These structures, endowed with high water-holding capacity, were combined with alginate biopolymers at varying viscosity levels. Subsequently, new hybrid biomaterials were developed using 3D printing technology, and cell-material interactions were investigated using human mesenchymal stem cells (MSCs) and L929 fibroblast cells.

MATERIALS and METHODS

Decellularization of Plant Tissues

In this study, the decellularization of succulent plant tissues was carried out before the production of hybrid scaffolds. The succulent plants were obtained from the greenhouse owned by the Faculty of Science at Ankara University. The samples were initially washed with an isotonic solution (NaCl 0.9%) at room temperature. Then, for the decellularization process, the plant tissues were rinsed in distilled water for an hour. Afterward, they were placed in different solutions for 72 hours. These solutions were 1% SDS solution, 3% SDS solution, 5% SDS solution, 1% Triton X-100 solution, 3% Triton X-100 solution, 5% TritonX-100 solution, and 1% SDS + 3% Triton X-100 solution respectively and the solutions were refreshed daily. Following this step, the tissues were immersed in an isotonic solution overnight. After their exposure to the isotonic solution, a series of steps were performed as follows: the tissues were submerged in ethanol for 3 hours, then in distilled water for 2 hours, and subsequently in a 3% H_2O_2 solution for 1 hour. After this, the tissues were left to soak in distilled water for one night. The next step involved immersing the tissues in a 3% sodium chloride solution for 2 hours. Upon completing this process, the tissues were rinsed in distilled water for one night. Following this process, the inner hydrochyme tissue was separated from the outer parenchyma using a scalpel. The separated tissues were frozen at -80 °C for 24 hours and subsequently lyophilized for another 24 hours.

Characterization of Decellularized Tissues

To determine the efficiency of the decellularization process, a commercially available plant-derived DNA isolation kit (GeneJet Plant DNA Isolation Kit) was used. The DNA content in both natural and decellularized tissues was measured and compared to determine the yield of decellularization. Furthermore, for protein determination after the decellularization process, the Coomassie Blue method was employed to measure the total protein content and compare it with the natural tissues.

Preparation of 3D Printed Hybrid Scaffolds

The hybrid tissue scaffolds were prepared by initially combining decellularized tissue scaffolds with a carrier polymer (alginate) to create 3D bioprinted scaffolds. Firstly, solutions with different viscosities were prepared using various alginates (A1-W201502 SAFC with a 1% aqueous solution viscosity of 5-40 cP at 25°C; A2-180947 Aldrich with a 1% aqueous solution viscosity of 15-25 cP at 25°C; A3-A7253 Sigma). These solutions were prepared at specific concentrations in water, and the 3D printability of these prepared samples was examined, as described in Jia et al. and Naghieh et al. [17, 18]. Furthermore, the rheological properties of the printing solutions prepared with different concentrations of alginate were examined in both plain and combined succulent samples using a basic rheometer.

7% alginate solution was prepared and mixed with 20% (w/w) succulent powder. (0.07 grams of alginate and 3.5 mg of succulent were used per 1 ml.) The solution was then homogenized to achieve uniformity, initially using a magnetic stirrer and then a homogenizer. During the gel printing process loaded onto the 3D printer, parameters such as the nozzle diameter, printing speed, and applied pressure were optimized in an attempt to achieve a satisfactory printing outcome. Four different groups were studied: group 1: 7% alginate + succulent I (Pressure: 57; Infill: 50%), group 2: 7% alginate + succulent II (Pressure: 45; Infill: 25%), group 3: 7% alginate + succulent III (Pressure: 45; Infill: 25 %), and group 4: 7% alginate.

Following the 3D printing process, the samples were subjected to a cross-linking process with 2% $CaCl_2$ (w/v) at room temperature for 2 hours and 24 hours, by the literature. After the cross-linking process, they were rinsed several times with D-PBS [19].

Characterization of 3D Printed Hybrid Scaffolds

The degradation behavior of the obtained tissue scaffolds was examined in terms of weight loss for up to 30 days in both enzymatic (lysozyme) and non-enzymatic (D-PBS) environments [20]. The initial weights of the tissue scaffolds were recorded, and the scaffolds were then subjected to degradation in separate PBS and lysozyme solutions at 37°C with a shaking speed of 75 rpm for 1, 3, 10, and 30 days. At the specified time points, the scaffolds were removed from the solutions, dried, and their weights were accurately remeasured.

In conjunction with the degradation behavior, the swelling behavior of the samples in a water environment was investigated. The swelling behavior of the samples over time was tested in distilled water, and the percentage of swelling was determined based on the following equation.

[(Ww-Wd) /Wd] *100 (Ww = post swelling weight; Wd = pre-swelling weight)

The structural examination of the final forms of the 3D-printed samples was conducted using Scanning Electron Microscopy (SEM). For this purpose, dry samples were gold-coated under vacuum conditions and then imaged directly using SEM (FEI, United Kingdom) at 10 kV.

The chemical structure characterizations of the prepared samples in their final structural forms were conducted using Fourier Transform Infrared Spectroscopy (ATR-FTIR; Perkin Elmer, USA) in the spectrum range of 4000-600 cm^{-1}. Additionally, structural characterizations were performed through X-ray diffraction (X-RD) analysis (Rigaku D/Max 2200 ULTIMAN). The analysis was carried out in an inert atmosphere with a scan rate of 5°/min at angles between 0° and 40°.

The potential influence of the plant-derived material on the thermal behavior of the tissue scaffold was evaluated using Thermogravimetric Analysis (TGA; (Pyris; Perkin Elmer, USA). Analysis of the samples was carried out in the temperature range of 25-800ºC, 10°C/min heating rate, and nitrogen environment. The weight change over time due to temperature variation was evaluated.

Cell-material Interactions

To investigate cell-material interactions in the hybrid tissue scaffolds prepared for the research, mesenchymal stem cells and L929 cells were employed. MSCs were cultured in a medium composed of DMEM High Glucose, 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine at 37 °C in a 5% CO_2 environment. In the culture of L929 cells used additionally for cytotoxicity tests, DMEM F/12 was used as the culture medium. Before all experiments, the samples were sterilized using 80% ethanol for 60 minutes and subsequently rinsed with sterile D-PBS.

The potential cytotoxic effects of the prepared tissue scaffolds (both normal and hybrid) were evaluated through an indirect MTT assay for up to 48 hours. For this purpose, cells were seeded in 96-well plates at a density of $1x10^3$ cells per well, and a standard MTT assay was applied. The results were assessed based on the control group (culture dish). The experiments were conducted with three repetitions. The MTT assay results were statistically evaluated using an analysis method called the ANOVA test, which is used to compare means among at least three groups.

In addition to the cytotoxicity studies on cells, the samples were subjected to Acridine Orange/Propidium Iodide (AO/PI) staining. For this purpose, a dye mixture prepared in a 1:1 (mM) ratio was used to stain the cells, which were washed with D-PBS. Following the staining and subsequent washing steps, the cells were examined with a fluorescence microscope.

RESULTS and DISCUSSION

Plant (Succulent) Tissue Decellularization and Its Effectiveness

Succulent samples were decellularized with different concentrations of SDS and Triton-X 100 chemicals. DNA analysis results from seven different concentrations of SDS and Triton-X 100 trials were presented in Table 1. All experiments at these concentrations were conducted in three replicates (n=3). The overall appearance of the succulent samples following decellularization is shown in Figure 1. As decellularization outcomes may vary across different tissues, distinct strategies could be pursued for each tissue.

In the literature, various detergents and surfactants are commonly used in singular or combined decellularization techniques. Additionally, as plant cells possess a cellulose-based cell wall, the ratios of chemicals used in decellularization studies on animal tissues, when adapted identically to plants, may not elicit the same decellularization effect. Therefore, the decellularization procedures were conducted using Triton X-100, an ionic detergent, along with the surfactant SDS.

While the chemical decellularization technique is relatively simple, due to plant cell structure, removing these applied chemicals from the cellular environment is a laborious and prolonged process compared to animal cells, as mentioned above. Thus, getting a high impact at low concentrations is the most desirable result. According to the analysis data in Table 1 (n=3), cellular

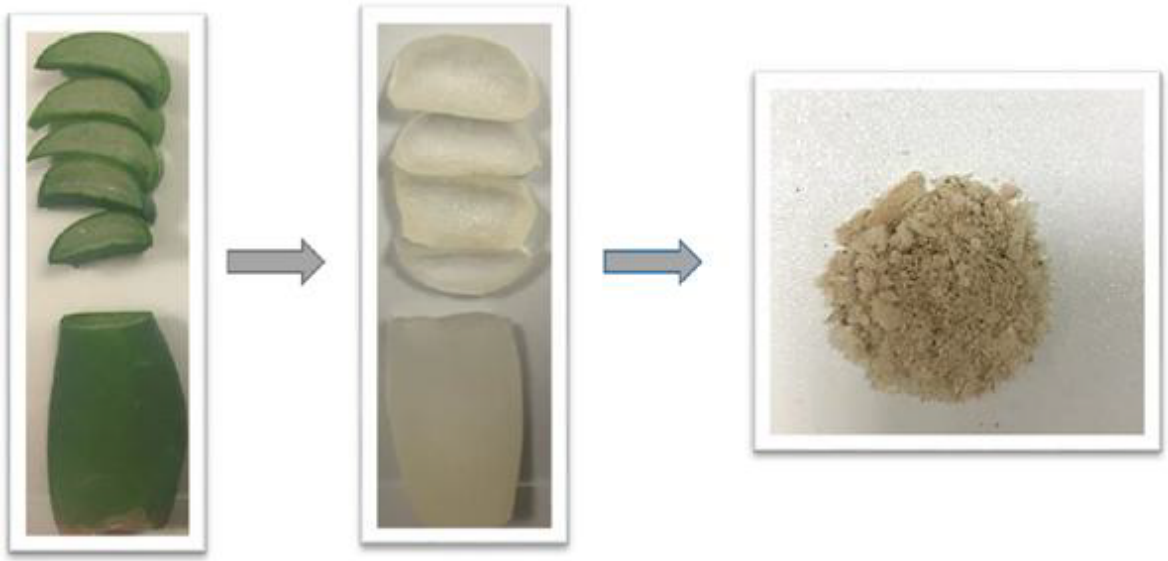


Figure 1. The decellularization steps of succulent plant.

DNA can be reduced more successfully with the ionic detergent Triton X-100. Despite the potential for a further decrease in DNA quantity due to increased concentration, subsequent washing procedures, and the possible hazardous and toxic effects of residues, the decellularization procedures continued with the 1% Triton X-100 group.

In contrast to animal tissues, plant tissues include cellulose and its derivatives within their cell wall structures. Furthermore, plant tissues are deprived of animal-derived proteins such as collagen and elastin, unlike animal tissue decellularization. Therefore, the preservation of protein content is not directly impactful on plant decellularization. Additionally, interference between Coomassie blue dye used for protein determination and plant sugars has been observed in the literature [21, 22]. This presented study aimed to analyze the effectiveness of decellularization rather than quantification. Consequently, in the selected 1% Triton X-100 group, results compared to the control indicated that the biochemical content in cells was significantly preserved (approximately 90.57 ± 1.20%).

Preparation and Characterization of 3D Printed Hybrid Scaffolds

3D printing has great potential for a wide variety of applications in the fields of bioengineering and tissue engineering. Alginate biopolymer was chosen as the carrier matrix of these hybrid scaffolds, and optimization and characterization of this process were achieved through a series of experiments and analyses. Smith et al. focused on the design of second-generation bioinks that would facilitate the identification of different alginate-polysaccharide bioink formulations and their optimal applications, allowing this relatively simple gel system

Table 1. The percentage of the DNA content reduction after succulent decellularization.

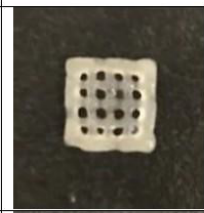
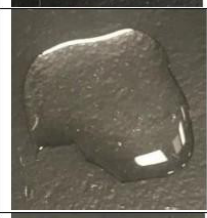
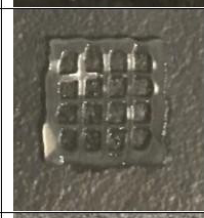
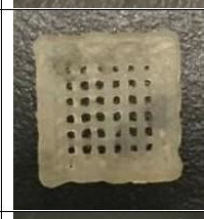
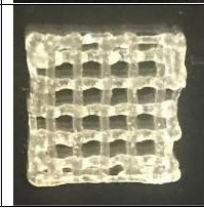
	Amount of DNA Reduction %
1% SDS	73,05 ± 9,60
3% SDS	75,74 ± 12,00
5% SDS	74,22 ± 13,60
1% Triton X-100	78,96 ± 3,06
3% Triton X-100	77,32 ± 4,34
5% Triton X-100	77,58 ± 5,30
5% Triton X-100 + 1% SDS	86,45 ± 1,42

to achieve more sophisticated control over biological processes. At the end of the study, alginate has been recognized as a biocompatible and ideal matrix for 3D printing [23]. One of the main reasons for choosing alginate as the carrier matrix was its proven biocompatibility and suitability for 3D printing. In the study, solutions were prepared with different alginate samples at different concentrations, and the printability of the prepared samples was examined. At this stage, no rheological quantitative evaluation was performed; direct printing experiments and a visual evaluation were applied. Some of the results are given in Table 2.

Rheological properties are critical factors in the 3D printing process. The concentration of alginate is one of the key parameters that determine these properties. The results of the study, show that alginate concentrations were most suitable for printing when they were in the range of 7– 10%. This concentration range exhibited the shear-thinning property and provided the viscosity reduction required for 3D printing (Figure 2). The shear-thinning feature is particularly important in the process of injecting ink or bioink, as smooth and repeatable printing is achieved by decreasing viscosity under increasing pressure. In their study in 2018, Wilson et al. stated that hydrogels such as alginate and agarose for 3D printers have the most suitable bioink viscosity characteristics and that this is obtained thanks to their shear-thinning properties. Rheological analyses in the study showed that viscosity decreased with shear-thinning properties, which is a critical factor for injectability in the 3D printing process [24].

Moreover, alginate concentrations were changed between 3-10%. During 3D printing, not only concentration but also many other parameters (pressure, infill rate, etc.) can affect printing. During printing, 20G, 21G, and 22G nozzle diameters were tested with pressure parameters between 20-45 psi and infill rates of 15-25%, respectively, and the most suitable parameters were optimized as nozzle diameter 21G, pressure 37 psi and infill rate of 25% (Figure 4).

Table 2. The images of 3D-printed alginate scaffolds at different concentrations.

Concentration of Alginate (w/v)	A1	A2	A3
3%			
5%			
7%			
10%			

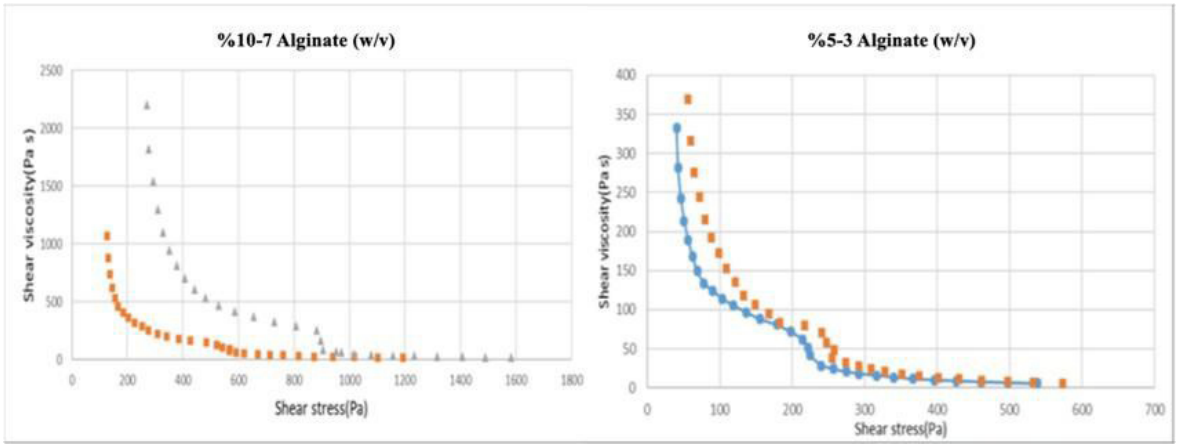


Figure 2. The rheological evaluations of alginate samples.

The basis of decellularization studies is based on the preservation of ECM structure and biochemical content. One of the most fundamental problems encountered here is that differences in different individuals of the same species directly affect the decellularization content. In other words, differences can be found from lot to lot. Similar problems have been encountered in different lots of the same species undergoing the same methodological decellularization processes (Figure 5).

The addition of decellularized powders from succulent plants may affect the biodegradation abilities of hybrid scaffolds. The examined biodegradation results showed that crosslinking time is critical in this process (Figure 6). In particular, a 2-hour crosslinking time led to rapid biodegradation of the materials. This is important to determine the structural stability of the hybrid material and may provide tunable biodegradation properties for future applications. Additionally, it is important to ensure porosity during the 3D printing process. The infill ratio is

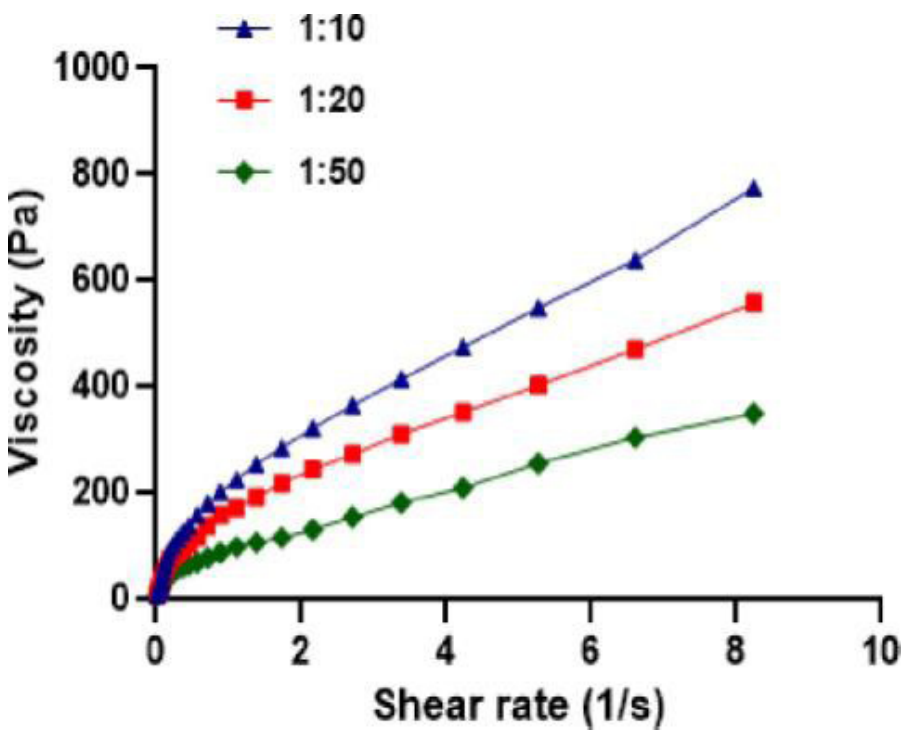


Figure 3. The rheological properties of samples prepared at different succulent concentrations.

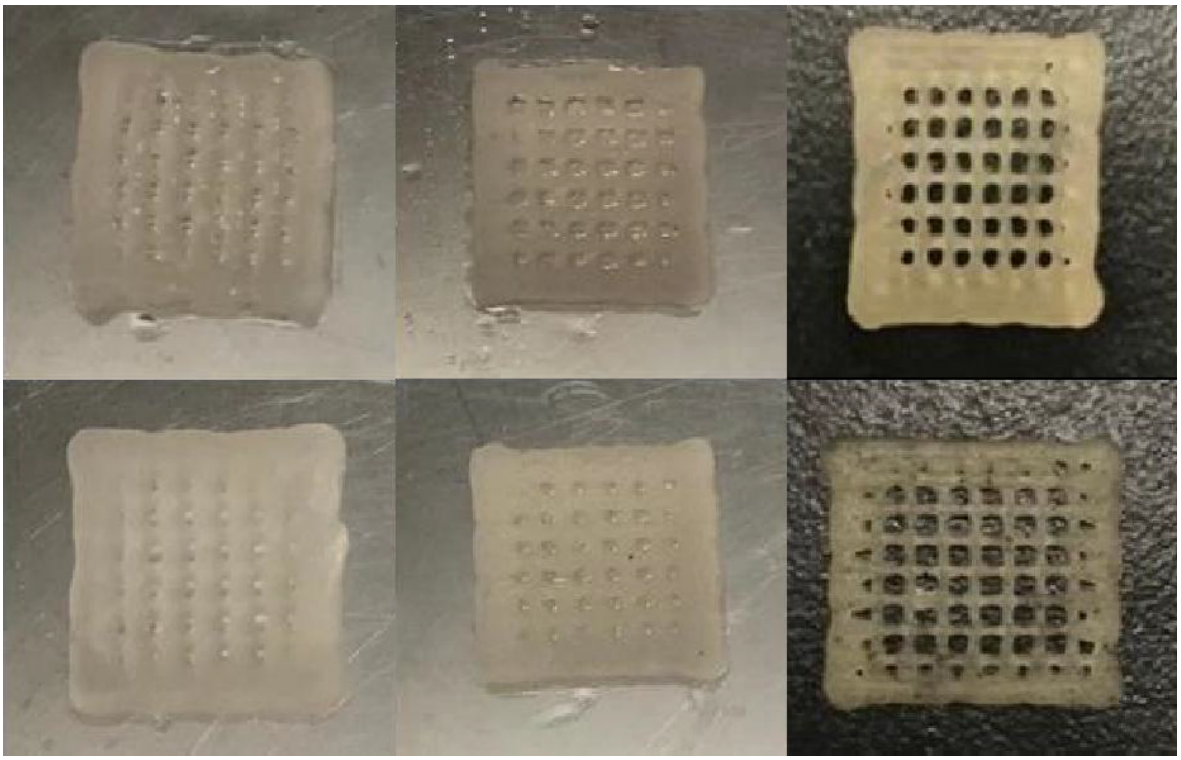


Figure 4. 3D-printed optimized samples at different alginate concentrations

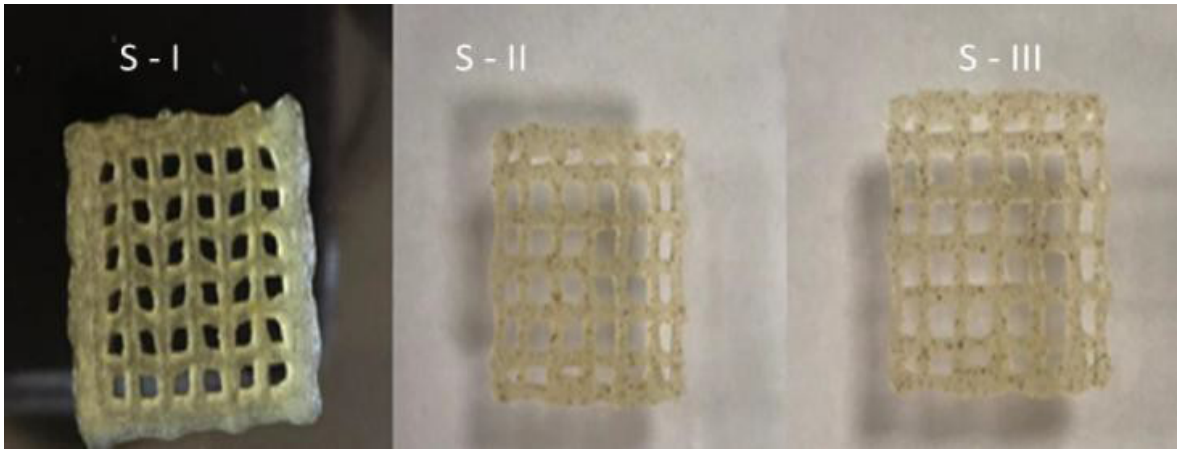


Figure 5. 3D-printed materials using different decellularized plant lots

Table 3. Time-dependent swelling behavior of 3D-printed alginate and hybrid samples (A: alginate; AP: hybrid) (n=3)

Swelling %	1 min	5 min
3A	126 ± 29	124 ± 14
3AP	179 ± 8	113 ± 14
7A	129 ± 25	164 ± 37
7AP	152 ± 38	141 ± 28

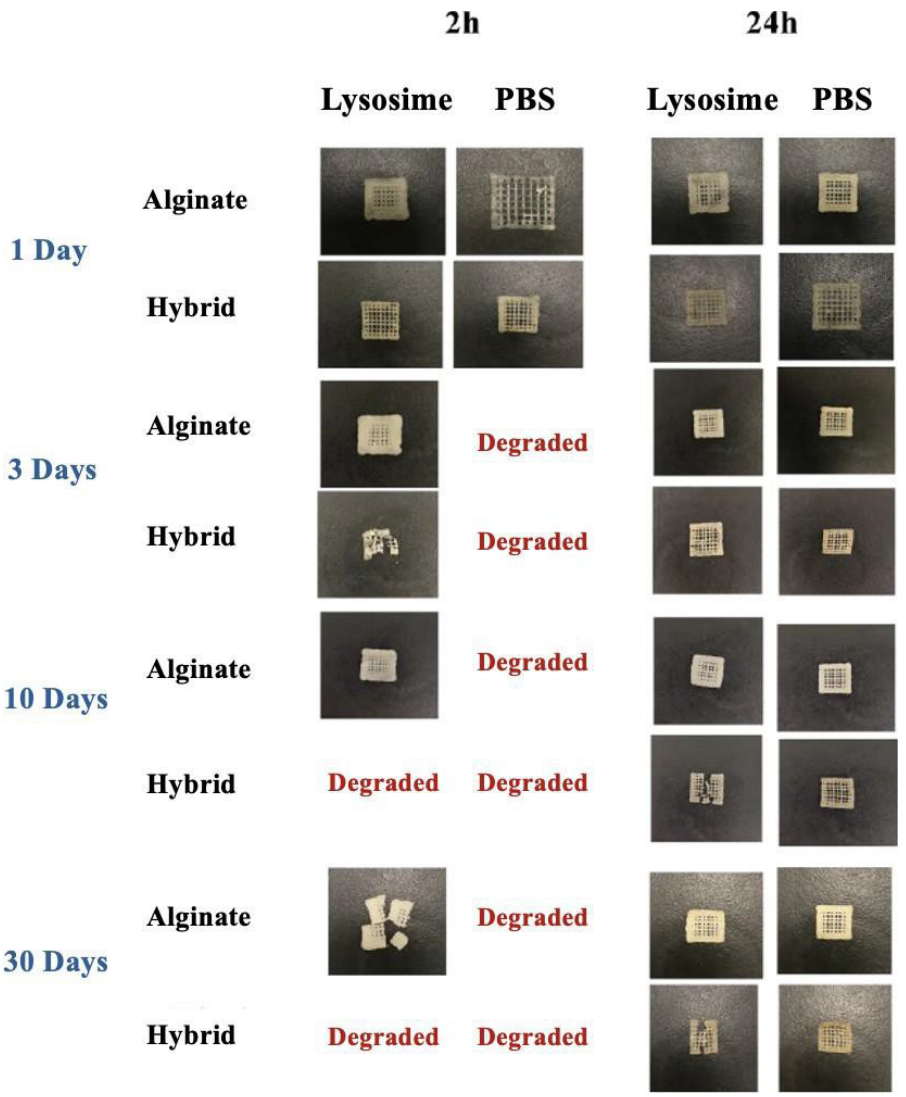


Figure 6. The degradation behavior of 3D-printed samples (only alginate and decellularized succulent/alginate) against time and at different cross-linking times and biodegradation environments (with and without enzymes).

a critical parameter affecting this porosity. High infill rates may result in scaffolds being more robust and compact, but this may limit water retention capacity. Thara and Kokol observed that the compressive modulus and stiffness of materials varied with wall diameters and crosslinking times. It is suggested that these parameters increase proportionally with the increase in crosslinking time and are inversely proportional to the pore sizes. In their study, the scaffold with smaller pores and crosslinking for a longer period provided superior modulus and stiffness even after 14 days of incubation in a physiological solution [25].

The swelling behavior of 3D-printed samples is also an important factor. Succulent content affects the water retention capacity of printed materials. This study showed that the water retention ability of decellularized succulent was lower than expected. This highlights the need for optimization to increase the water retention capacity of scaffolds in future applications.

In the SEM images given in Figure 7, it was seen that 3D printing created a certain macro porosity within the infill value. In the samples with a pore size of 300-350 microns, as a result of the studies on increasing the infill value, proper printing could not be obtained with the printer we have at each application value at the thinner nozzle. At the same time, interlayer collapses were observed.

Structural characterization analyses have provided important, insightful information about the composition

and thermal behavior of hybrid material. X-ray diffraction (XRD) analysis was successful in isolating the chemical structures of alginate and succulent, identifying the unique chemical properties of both components. This analysis was effective in determining the characteristic structure of alginate, especially the presence of certain chemical groups such as manuronic acid and uronic acid (Figure 8).

Furthermore, as shown in Figure 9, Fourier transform infrared spectroscopy (FTIR) analysis demonstrated the structural similarities of alginate and succulent and helped us better understand the chemical relationship between the two components. In particular, the similarities between manuronic acid in alginate and certain chemical groups in the succulent were highlighted by this analysis. The absorbance peaks of manuronic acid and uronic acid were observed at 880-900cm^{-1} and 935-940cm^{-1} in the structure of alginate, respectively. In addition, the beta-glycosidic bond band between glucose units from cellulose was found at approximately 890 cm^{-1}. It was evaluated that these structures overlap in

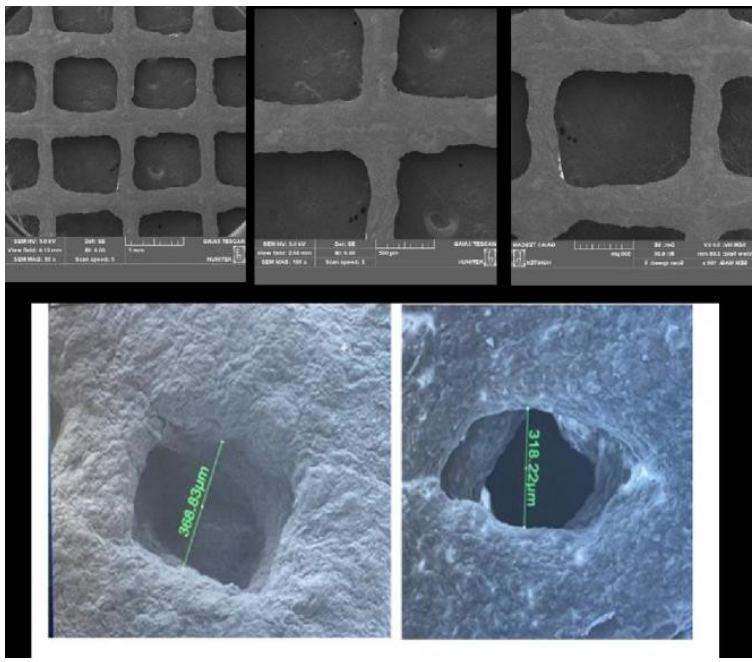


Figure 7. SEM micrographs of 3D-printed samples.

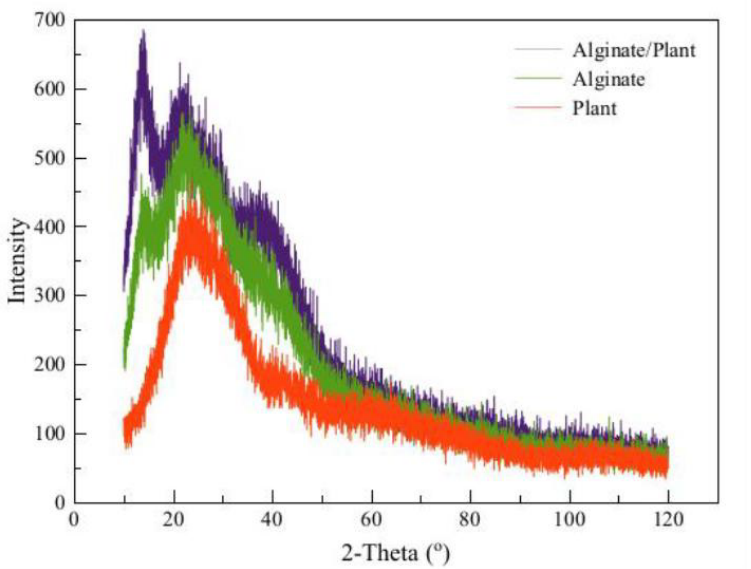


Figure 8. XRD Spectrum of precursors of 3D-Printed samples.

hybrid samples (B) [26, 27].

In the comparative TGA analyses (Figure 10) on 3D-printed samples, it was concluded that the introduction of the hybrid material into the structure made a difference in the final remaining mass amount in the evaluations made based on weight loss %, but did not create a significant difference in the thermal behavior of the samples.

Cell-Material Interactions

When cells were seeded on 3D-printed materials prepared with different concentrations of alginate and hybrid materials containing succulents, it was observed in the direct MTT test that the cells detached from the surface and floated within the first 24 hours. The experiment was repeated twice. Since the same results were obtained in both experimental repetitions, it was deemed appropriate to coat the samples with collagen as it could facilitate cell adhesion. To realize this, the samples were treated with 100 microliters of sterile 0.1% collagen solution for 30 minutes in a laminar cabinet. All MTT cytotoxicity tests were repeated with collagen-coated 3D-printed samples. Additionally, in the cytotoxicity studies,

L929 fibroblast cells specified in the ISO-10993 standard were used in addition to the mesenchymal cells in the research [28]. The results are presented in Figures 11 and 12, respectively.

The MTT assay is a standard cytotoxicity evaluation method applicable through both direct and indirect (elution) approaches. This assay relies on the reduction of the yellow MTT dye to formazan crystals by the enzyme alcohol dehydrogenase present in the cells. In this study, the direct MTT assay involved seeding cells into a culture plate, followed by the addition of samples of equal volume to the wells. In contrast, the indirect (elution) MTT assay involved treating the culture medium with samples, which was then applied to the cells. Both assay methods revealed that the samples exhibited varying degrees of cytotoxicity towards the cells. Although some recovery was noted in specific groups at the 48-

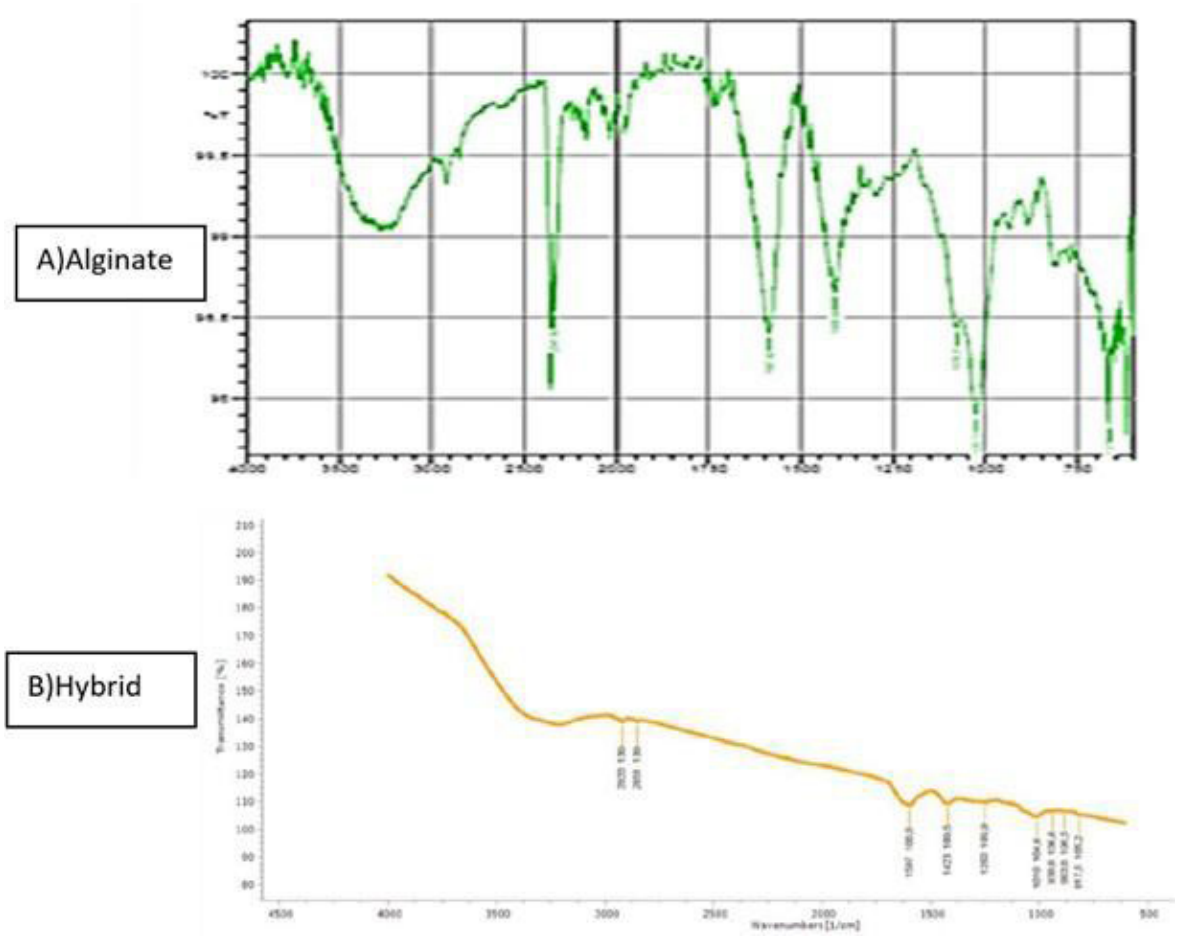


Figure 9. FTIR spectra of 3D-printed samples.

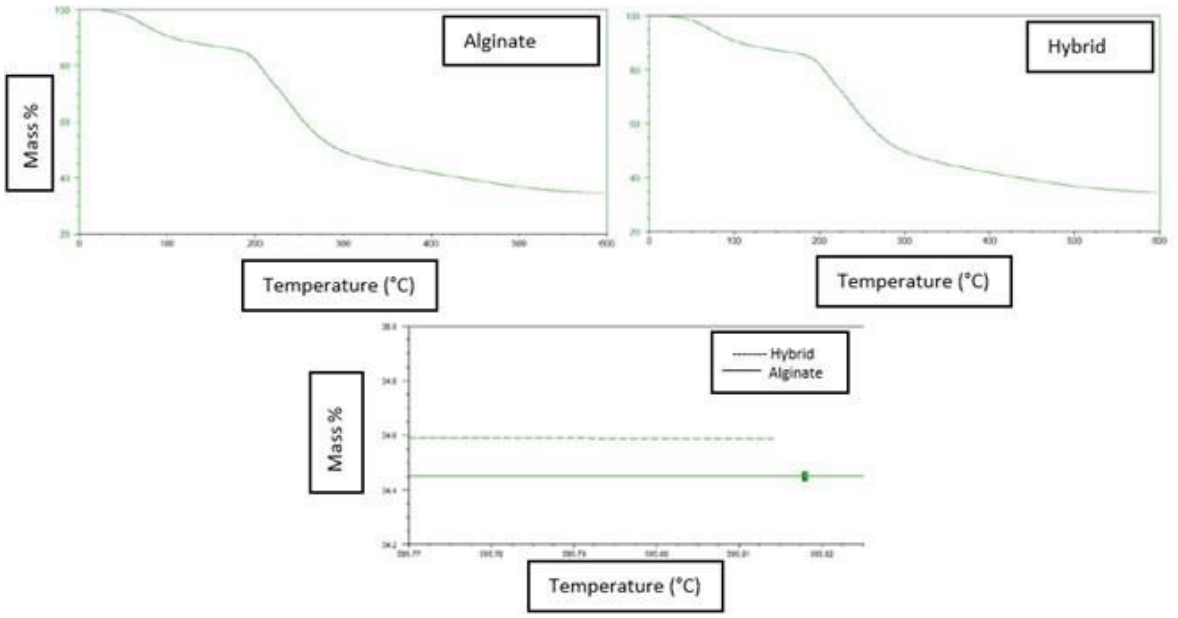


Figure 10. TGA analysis thermograms of 3D-printed samples.

hour mark, this was interpreted as cellular proliferation activity. When compared to the control group, a significant reduction in cell viability was observed across all experimental groups. For instance, both the 24-hour and 48-hour results indicated a marked decrease in cell viability for 3D-printed samples incorporating mesenchymal stem cells within succulent groups containing 5% and 7% alginate (with P-values of 0.0278 and 0.0066, respectively, compared to the control). Additionally, in experiments involving L929 fibroblast cells, a significant decrease in cell viability was observed in the 5% and 7% alginate succulent groups relative to the control (with P-values of 0.0004 and 0.0001, respectively).

In tests conducted with mesenchymal stem cells, although there was a decrease observed in the hybrid group containing 3% alginate compared to the control, this decrease was not found to be statistically significant. However, in tests conducted with L929 cells, this result was considered significant (P=0.0142).

Upon analyzing the results from the direct MTT assays, a statistically significant reduction in cell viability was noted in hybrid tissue scaffolds containing succulents when compared to the control. This decrease was observed for both mesenchymal stem cells and L929 fibroblast cells at 24 and 48 hours. Specifically, for scaffolds with mesenchymal stem cells, the P-values for 5% and 7% alginate were 0.0121 and 0.0437, respectively; for L929 cells, the P-values were 0.0273 and 0.0288, respectively.

These findings suggest that, in addition to the reduced viability associated with increased alginate concentration, the inclusion of succulent content in hybrid structures exacerbated cell viability loss, as indicated by both direct and indirect measurements. Particularly, after 48 hours, viability decreased to 50% in groups containing decellularized succulents.

To further investigate the observed low cell viability at 24 and 48 hours, a proliferation study using AlamarF Blue was conducted over a period of up to 10 days. In this phase, cells were directly seeded onto the samples (50 x 10^3 cells/sample) using non-binding culture plates. Contrary to the MTT results, this experiment yielded inconclusive outcomes after the first day. Cells were observed to be floating in the culture medium, and fragments of succulent material were also present in the medium. This occurrence was interpreted not as a result of material degradation but rather as erosion from the sample surface.

Following this stage, due to the inconvenient interaction between the cells and the material, an alternative approach was attempted. As previously mentioned, the MTT test evaluates cell viability based on enzyme activity. Here, even under specific stress conditions, as long as alcohol dehydrogenase enzyme activity continues, the cells are considered alive. However, according to the progress of the experiment, the cells should have continued to show viability even after 48 hours. Des-

pite this, living cells were not observed from the proliferation test. Consequently, after 48 hours, a possible Acridine Orange / Propidium Iodide (AO/PI 1:1) staining was performed to observe the necrotic activities of the cells. For this purpose, the cells were stained with AO/PI, and their conditions were observed using a fluorescent microscope.

In the AO/PI staining analysis presented in Figure 13, live cells were stained green, apoptotic cells appeared yellow-orange and non-viable or necrotic cells were stained red. The analysis of the results revealed an increase in both apoptotic and necrotic activity within the first 24 hours across all experimental groups compared to the control. This increase was attributed to the concentration of alginate and the presence of decellularized succulents. Despite these observations, the overall cell viability remained relatively stable during this initial period.

However, after 48 hours, a pronounced decline in cell viability was observed in all experimental groups compared to the control. While cells retained a certain level of viability initially, the incorporation of decellularized succulents, particularly at higher alginate concentrations, led to a more significant loss of viability. Although reducing the succulent concentration resulted in some improvement in cell viability, it was deemed insufficient for the cells to sustain viability beyond 48 hours.

The most extensively studied and reported species within the succulent plant family, concerning the study, can be exemplified by Aloe Vera plants, even though they are not directly used in the study. Aloe Vera plants consist of nearly 98% water, with the remaining portion comprised of carbohydrates, amino acids, minerals, and other macromolecules. Studies in the literature have reported cytotoxic effects of succulent plant extracts on various cancer cells, much like in many other plant extract studies. For instance, research by Abdul-Hafeez et al. demonstrated the cytotoxic effect of methanolic extracts from specific succulent plants on colon, breast, and liver cancer cells [29]. In the study conducted by Du Plessis et al., a dose-dependent increase in apoptosis was observed in Hela and Hep2 cells with concentrations up to 1000 mg/mL [30]. Furthermore, studies have

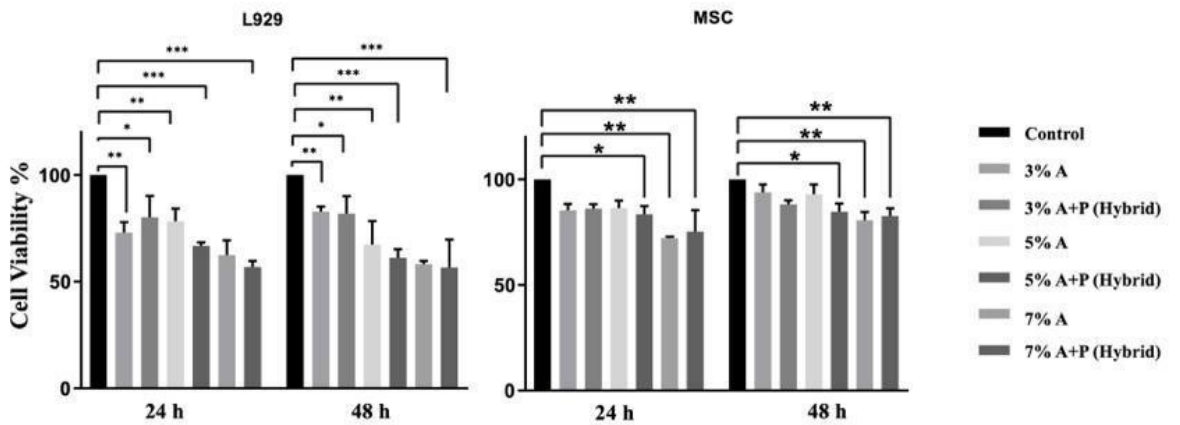


Figure 11. Indirect (Elution) MTT test against MSC and L929 cells at 24 and 48 hours (n=3) (* = P ≤ 0.05; ** = P ≤ 0.01; *** = P ≤ 0.001)

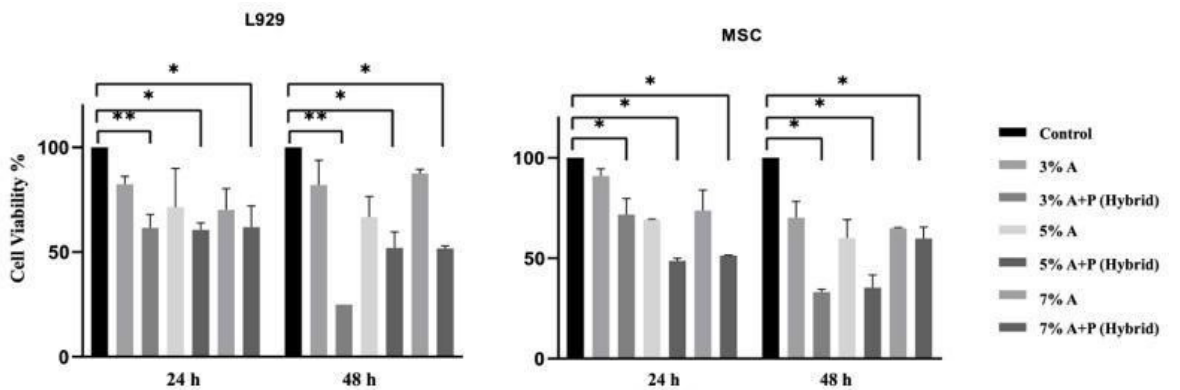


Figure 12. Direct MTT test results against L929 and MSC cells at 24 and 48 hours (n=3) (* = P ≤ 0.05; ** = P ≤ 0.01; *** = P ≤ 0.001)

suggested that Aloe Vera extracts (in full leaf, gel, or latex forms) might exhibit certain levels of carcinogenic and cytotoxic effects [31]. It is evident that current literature studies primarily progress through extracts, and these extracts can enforce toxic effects on cells at specific concentrations. In 3D printing and scaffold formation involving plant extract blends, such as the study by Ayran et al., it was reported that fibroblast cell viability decreased with increased extract quantity and culture duration in 3D-printed PCL scaffolds containing juglone (walnut extract) [32].

In this research, the intention was to prepare a scaffold using 3D printing technology by combining the powdered form of succulent structure obtained through decellularization from the whole leaf (internal structure) and combining it with a biopolymer-based carrier (alginate). In this context, the succulent used was directly in the form of powdered whole decellularized leaves, rather than an alcoholic extract added to the mixture. It is particularly considered that the cytotoxicity effect is exhibited by the direct addition of the decellularized succulent structure in powder form without undergoing any additional biomolecule purification processes.

CONCLUSION

This study focuses on creating scaffolds for tissue engineering using 3D printing and decellularization techniques. Various decellularization methods were employed to assess the water-holding capacity of succulent plant parenchyma tissues. The decellularized succulent plant tissues were then combined with alginate, a widely used biopolymer for 3D printing. The physical and chemical characterization of control and hybrid materials was performed during 3D printing, considering the conditions of the printing process.

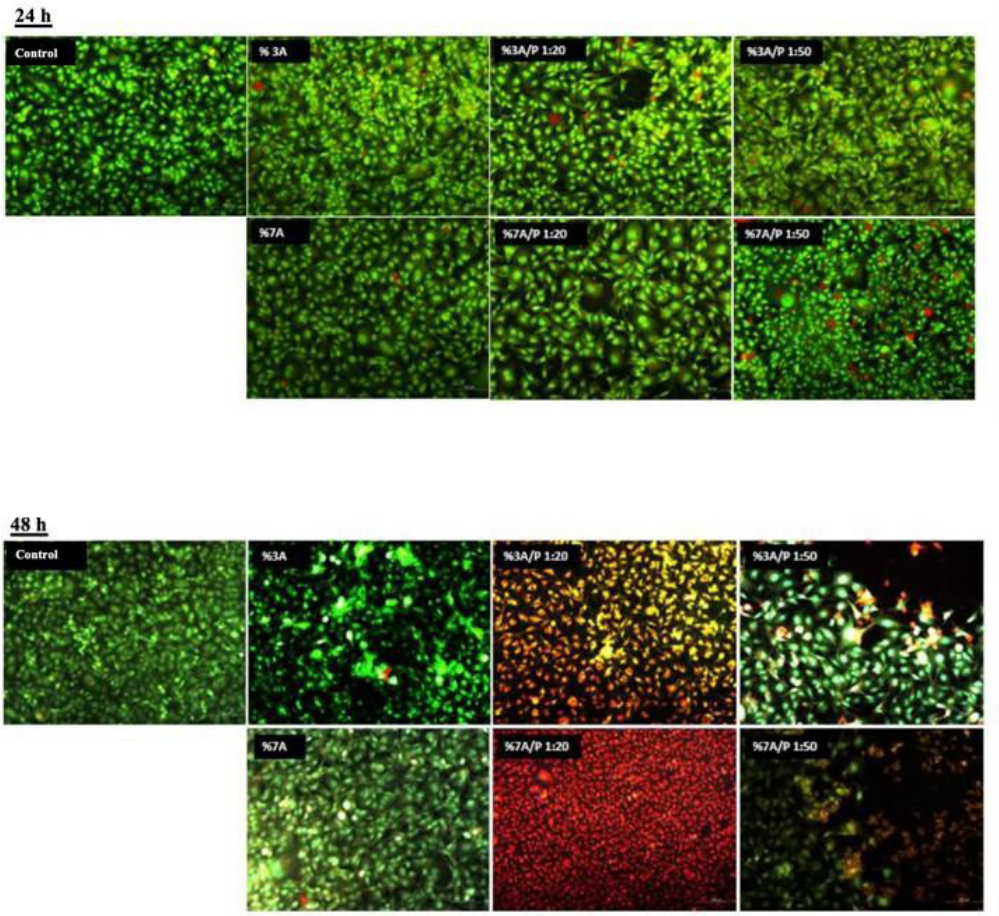


Figure 13. AO/PI staining in MSC cells interacted with 3D-printed materials (A: Alginate, A/P: Alginate/Plant).

Analysis using L929 fibroblast cells and mesenchymal stem cells revealed that all printable hybrid scaffolds exhibited deleterious effects, leading to a loss of cell viability after 48 hours. The significance of this decrease in viability, compared to the control group, is notable. Specifically, it was found that the decellularized succulent powders were the main cause of the observed toxicity when added directly to the mixture at specific concentrations, and even after cross-linking the 3D-printed structures. This led to a loss of structural integrity in the culture medium.

The results suggest that further improvements are needed in the design and fabrication of hybrid materials. Based on the obtained data and observations, the following recommendations for advancing the research are proposed: Particularly when the alginate concentration is low, cell viability appears to be more sustainable. In cases where the existing infrastructure cannot support 3D printing, samples with less than 3% alginate should be prepared using a higher-resolution 3D printer. To minimize variability between batches of the biopolymer carrier and address biocompatibility issues, experiments should also be conducted using more biocompatible carriers, such as collagen or gelatin. Additionally, to achieve the goal of utilizing the water-retention properties of succulent plants, interdisciplinary projects involving standard succulent species are necessary. Although this work represents an important advancement in the field of plant tissue decellularization, it requires further research and development.

Regenerative medicine is a field focused on repairing or regenerating damaged or lost cells, tissues, and organs. When organ transplantation is not feasible or there is a shortage of organ donations, the development of artificial organs becomes necessary. In this context, scaffolds that mimic the organ to be repaired are produced in the laboratory, with tissue engineering playing a crucial role in this process. One method used to prepare these scaffolds is decellularization. Decellularization is an effective approach for obtaining 3D biomaterials composed of the extracellular matrix (ECM), which provides biomechanical support in tissues, particularly for 3D printing. Tissue engineering and regenerative medicine have increasingly adopted this procedure to prepare suitable scaffolds for tissue repair. Plant tissue decellularization is one of the most commonly used methods for obtaining scaffolds. Additionally, it is environmentally friendly and more abundant compared to human and animal-derived materials. The succulent plant used in the study was chosen due to the gelatinous substance in its parenchymal tissue and its high-water retention capacity, which are advantageous for creating 3D materials. Literature shows that succulent plants are utilized in tissue engineering studies for their water retention capacity. The aloe vera plant, a succulent, demonstrates significant potential as a source of scaffolds, gels, and films in tissue engineering due to its water retention ability [33]. Aloe vera parenchymal tissue has been employed to produce collagen/chitosan/aloe vera gels, with evaluations concerning wound healing and tissue regeneration [34]. Ongoing global research on 3D-printed decellularized hybrid scaffolds offers promising prospects for creating desired tissues and organs and for personalized designs. Although direct applications of succulent plants in regenerative medicine are currently limited, the decellularization method used in this study, along with the optimization of alginate as a carrier biopolymer and the required concentration ratios for 3D printing, is expected to make these scaffolds a promising biomaterial for future use in 3D printing. This work could be part of an ongoing investigation into the decellularization of plant tissues and their potential applications in cell-material interactions and as scaffolds for tissue engineering. Based on these findings, further research should be conducted to better understand how hybrid scaffolds which contain alginate and decellularized succulents affect the differentiation of mesenchymal stem cells into tissue cells. Additionally, considering the mechanical properties, a compression test may be conducted to evaluate the scaffold strength. A theoretical approach can be made to reflect the natural water-retaining structure of cartilage tissue by using the water-retaining properties of the succulent plant, which exhibits water-retaining properties in nature. However, the toxic contributions of the succulent plant to the hybrid tissue scaffold may limit this approach. Additionally, since succulent plants vary from lot to lot, new interdisciplinary projects using standard succulent species are needed.

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Conflict of Interest

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript

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