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Decellularized leaf-based biomaterial supports osteogenic differentiation of dental pulp mesenchymal stem cells

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Abstract
Decellularized tissues are an attractive scaffolds for 3D tissue engineering. Decellularized animal tissues have certain limitations such as the availability of tissue, high costs and ethical concerns related to the use of animal sources. Plant-based tissue decellularized scaffolds could be a better option to overcome the problem. The leaves of different plants offer a unique opportunity for the development of tissue-specific scaffolds, depending on the reticulate or parallel veination. Herein, we decellularized spinach leaves and employed these for the propagation and osteogenic differentiation of dental pulp stem cells (DPSCs). DPSCs were characterized by using mesenchymal stem cell surface markers CD90, CD105 and CD73 and CD34, CD45 and HLA-DR using flow cytometry. Spinach leaves were decellularized using ethanol, NaOH and HCL. Cytotoxicity of spinach leaf scaffolds were analysed by MTT assay. Decellularized spinach leaves supported dental pulp stem cell adhesion, proliferation and osteogenic differentiation. Our data demonstrate that the decellularized spinach cellulose scaffolds can stimulate the growth, proliferation and osteogenic differentiation of DPSCs. In this study, we showed the versatile nature of decellularized plant leaves as a biological scaffold and their potential for bone regeneration in vitro.

Keywords Stem cell · Osteogenic differentiation · Plant scaffold

Introduction

Tissue engineering has become a part and parcel of biomedical research. In tissue engineering, biomaterials need to be bio-compatible to support regeneration of tissues. Various biomaterials (natural and synthetic) have been proposed and are still under investigation to achieve the appropriate morphological, physical, mechanical and biological properties suitable for the regeneration of targeted tissues (Prete *et al*. 2023).

Stem cell–based tissue engineering begins from cells to the injured tissue or blood vessel. In regenerative medicine, translational research, such as tissue engineering and molecular biology, is concerned with the process of replacing, or repairing cells, tissues or organs in order to resume organ function. However, it is hard to track the transmitted cells and keep them in a specific location. Scaffolds are the central components that are used to carry the cells, drugs and genes into the body. Various types of scaffolds are prepared as typical 3D porous matrix, nanofibrous matrices or porous microspheres, which provide suitable substrates for cell attachment, cell proliferation, differentiated function and cell migration (Eltom *et al*. 2019). Scaffold matrices have a specific advantage in regenerative medicine. Nature-developed plant-based scaffolds are a new technology of applied science for stem cell transplantation (Bružauskaitė *et al*. 2016). The varieties of natural polysaccharides and protein have been explored for bone regeneration (Ardeshirylajimi and Hosseinkhani 2013).

Scaffold designing that allows for the structural and functional repair of the bone remains a major task. Our aim is to develop decellularized plant-based scaffolds to support bone regeneration. Bone is made up of a hard and dense type of connective tissue with excellent mechanical properties (Weatherholt *et al*. 2013). It supports the human body, and stores and releases minerals. It contains osteoblasts,

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osteoclasts, osteocytes and bone lining cells embedded in the extracellular matrix (ECM). Osteoblasts produced mineralize for new bone matrix, and repair and regeneration of bone. Dental pulp stem cell is an ideal candidate for bone regeneration. Dental pulp stem cells (DPSCs) are possible to isolate from the extracted human permanent third molar pulp. These cells have characteristics as MSCs and fibroblast-like morphology. DPSCs have high proliferation rates, are clonogenic and possess all properties of stem cells (Patil *et al*. 2018a). DPSCs are multipotent and can differentiate into neural, adipocyte, odontoblast, etc. (Nuti *et al*. 2016). Dental pulp stem cells have the potential to differentiate in functional osteocyte. DPSCs secrete growth factor (Shekatkar *et al*. 2022), cytokinin (Bari *et al*. 2019) and scaffold which serves as a temporary platform that provides structural support, facilitates bone repair and guides bone growth in bone defects.

Plant leaves are structures developed by nature, which can be applied to tissue engineering. Spinach leaves have been the ideal example of plant-borne scaffolds. Various techniques for the development of plant-based scaffolds, like apple-derived cellulose scaffolds, spinach, bamboo sparges, carrot, celery, cucumber, potato, asparagus, green onions, leek and broccoli, have been employed by researchers (Bilirgen *et al*. 2021). Scaffolds are prepared to influence the physical, chemical and biological environments of a cell population (Howard *et al*. 2008). The requirement for unique scaffold structure and reproducible manufacture of these approaches results in improved scaffolds that are employed for cell development.

In tissue regeneration, a scaffold should be mechanically stable as well as biodegradable. Its size should be appropriate and it should have a rough surface and porosity which is required for providing a suitable microenvironment for sufficient cell–cell interaction, cell migration, proliferation and differentiation. Pore size plays a major role in cell adhesion, cell-to-cell interaction and other transmigration across the membrane based on the purpose of tissue regeneration (Bružauskaitė *et al*. 2016; Lee *et al*. 2022). The scaffold biomaterials should be non-toxic to humans, resistant to quick degradation and with the corresponding pore size or porosity (Krishani *et al*. 2023). A lot of properties that are needed for biomaterial design are expressed in the structure as well as the function of plants (Fontana 2019). It is also shown that decellularized plant tissue can be used as an adaptable scaffold for culturing human cells by simple bio-functionalization technique; it is possible to qualify the adhesion of human cells on various sets of plant tissue (Fontana 2019).

The hydrophilicity and prime water transfer qualities of plant tissue allow cell expansion. The microstructure of the plant frameworks, cell alignment and shape registration are unique physical characteristics, and the ability to manufacture biomaterials with a range of attainable physical and biological properties is left as a challenge and is an active area of decellularization (Fontana 2019).

Decellularized animal tissue such as human amniotic membrane has been used for a long time (Abazari *et al*. 2020; Lakkireddy *et al*. 2022). Plant tissue decellularized scaffold may reduce availableness problems, elevated costs and ethical concerns associated with animal sources. Tissue engineering needs a precise design of engineered biomaterial which is able to assist in the regeneration of misplaced or lost tissues. Fabricated as well as naturally derived biomaterials have been suggested and are still under inspection to accomplish the correct or appropriate mechanical as well as morphological, physical and biological properties to fulfill particular demands for the regeneration of target human tissues (Contessi Negrini *et al*. 2020).

The generation of vegetal scaffolds by dissociation of plant-based biomaterials has seen an increase in recent years. They are cost-effective and sustainable since the vegetal tissues are obtained from plant leaves, stems, fruits and vegetables. Previous studies have shown that decellularized spinach leaves scaffold are vascularized, which supports mammalian cells (Fontana 2019). Spinach leaves are cost-effective and free from animal-derived components. Most of the biomaterial scaffolds used in tissue engineering are of animal origin such as chitosan and collagen. The leaf surface is covered with a cuticle layer which makes a surface smooth known as the epicuticular wax. Most of the literature suggests use of acids (HCL and nitric acid) to remove the leaf epicuticular wax (Holloway and Baker 1968). This study evaluated viability and differentiation potential of DPSCs. Based on the evidence provided by the European Parliament's joint motion for innovation to minimize and eliminate animal use and promote plant-based material, a majority of studies focus on utilizing plant tissues to generate scaffolds for tissue engineering (Harris *et al*. 2021). Our aim to investigate the spinach leaf scaffold supports the osteogenic differentiation; it can be used in regenerative medicine for bone regeneration and repair.

Materials and methods

Selection of leaf samples Spinach leaves were selected for the study. Fresh spinach leaves were collected from a local supermarket washed with PBS and stored at 4℃ for a maximum of 2 days before use.

Decellularization of the leaf The leaves were cut longitudinally or transversely into 1–2-mm-thick slices. The leaves were boiled and then the leaf samples were treated with three chemicals, i.e. ethanol, sodium hydroxide and hydrochloric acid. Scaffold A was treated with ethanol, sodium hydroxide and 5% hydrochloric acid. Scaffold B was treated

with ethanol, sodium hydroxide and 1% hydrochloric acid and kept under UV light sterilization for 30 min (Dai *et al*. 2016). Cell adherence on surface is observed by using a phase contrast microscope. For further confirmation, cells were stained with fluorescent antibodies and confirmed by confocal microscopy.

Cell isolation from dental pulp tissue Stem cell isolation method was studied which was approved by an Institutional Stem Cell Committee for stem cell research. Mesenchymal stem cells were isolated from dental pulp which are also known as dental pulp mesenchymal stem cells. Tissue was obtained from Dr. D.Y. Patil Dental College and Hospital Pune. Tooth were extracted with prior consent from a healthy donor during dental procedure. The extracted tooth was cut vertically to remove the pulp by using air rotor. Dental pulp was cut into 1–2 mm size placed in culture dish in the presence of fetal bovine serum (FBS) and incubated for 24 h in CO_2 incubator. After 24 h, the completed media were added in culture dish for cell outgrowth. Cells were cultured by explant culture method (Patil *et al*. 2018b). DPSCs were characterized by using stem cell surface markers CD90, CD105, CD73 CD34, CD45 and HLA-DR using flow cytometry.

Cell seeding on plant-based leaf scaffold To maintain pH, leaf scaffold was washed with media and PBS. After maintaining pH, DPSCs (1×10^5 cells/well) were seeded on the leaf scaffold for attachment and bone regeneration in 24-well plates. Then the cells were incubated in 5% CO_2 incubator at 37℃ and we let the cells proliferate on the leaf scaffold.

Confocal microscopy and scanning electron microscopy to confirm cell growth Verification of cell growth on the scaffold was done by using antibody-specific staining DAPI and CD90PE for stem cells and the cells were visualized under confocal microscopy, and further cell growth confirmation was done by scanning electron microscopy (SEM).

Differentiation The DPSCs were cultured (1×10^6 cell/well) on the spinach leaf scaffold. These were subjected to osteogenic differentiation by inducing the DPSCs with osteogenic induction media containing 1 mM dexamethasone, 1 mM ascorbic acid and 0.1 mM β-glycerolphosphate use as positive control. Cells were cultured on plant-based scaffold and induction was done using osteogenic induction media. Cells were incubated for 18 to 21 d, and induction media were replaced twice a wk. Differentiated cell mineralization was stained using alizarin red. Cell mineralization was dissolved in 0.1% acetic acid for quantitative analysis and measure was taken at 450 nm on Elisa reader.

All experiments were performed in triplicate.

Gene expression Total RNA was isolated using an RNA isolation kit according to the manufacturer's protocol. The RNA level and quality were checked using the Qubit Nanodrop technology (Thermofisher, Waltham, MA). A total of 500 ng of RNA was used for reverse transcription using the Superscript III reverse transcription kit (TAKARA Kusatsu, Japan). The quantitative PCR analysis was performed using a QuantStudio 5 real-time PCR system (Thermo Fisher Scientific) and TaqMan gene expression qPCR Master Mix (Thermo Fisher Scientific) following the manufacturers' instructions. The primer sequences used were osteonectin. GAPDH were used as housekeeping gene (Table 1).

Result

Isolation of dental pulp stem cells Mesenchymal stem cells were successfully isolated by using the explant culture method. The outgrowth of cells were observed after 1 week of explant culture (Fig. 1*A*, *B*). MSCs show fibroblastic structure (Fig. 1*C*).

DPSCs show mesenchymal stem cell properties. DPSCs were positive for CD90, CD105 and CD73 and negative for CD34, CD45 and HLA-DR surface marker (Fig. 2). DPSCs also show trilineage differentiation properties such as osteogenic, chondrogenic and adipogenic differentiation. In osteogenic differentiation (calcium deposition), stain by alizarin red, chondrogenic differentiation (glycosaminoglycan) stain by alcian blue and adipogenic differentiation (lipid droplet) stain by oil red O stain were used (Fig. 3).

Cell attachment on plant-based scaffold Selection of leaf was done and spinach leaf was selected due to its structure. As spinach leaves have a hierarchical structure with veins interspersed with pores, their structure provides a scaffold that stimulates cell growth and organization. The scaffolds were chlorophyll free by using 99% of ethanol and NaOH (Fig. 4*B*, *C*). This chlorophyll-free plant-based scaffold is decellularized by using sodium hydroxide to make it transparent. The cuticle of the leaf was removed by using HCl and they appear completely white. Porous leaf scaffold was

Table 1. Primer sequence

Gene	Sequence	Length
GAPDH	Forward primer-TTTTGCGTC GCCAGCC Reverse primer-ATGGAATTT GCCATGGGTGGA	261
Osteonectin	Forward primer-TCGGCATCA AGCAGAGTGAG Reverse primer- CGATATCCT CTGCAAAGCAAGA	143

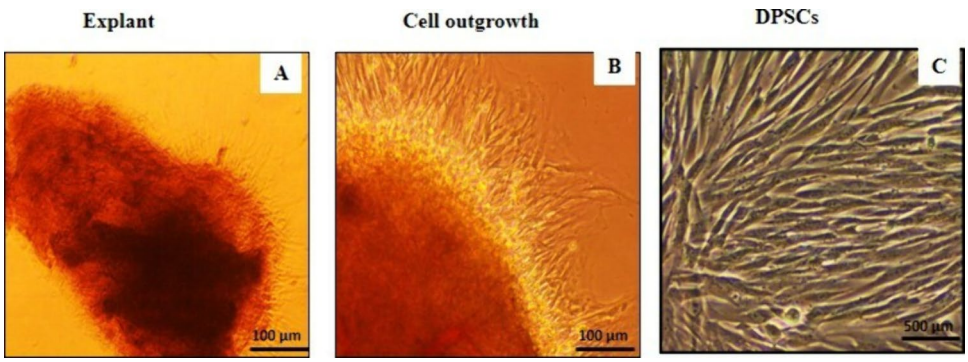


Figure 1. Isolation of DPSCs by using explant culture method.

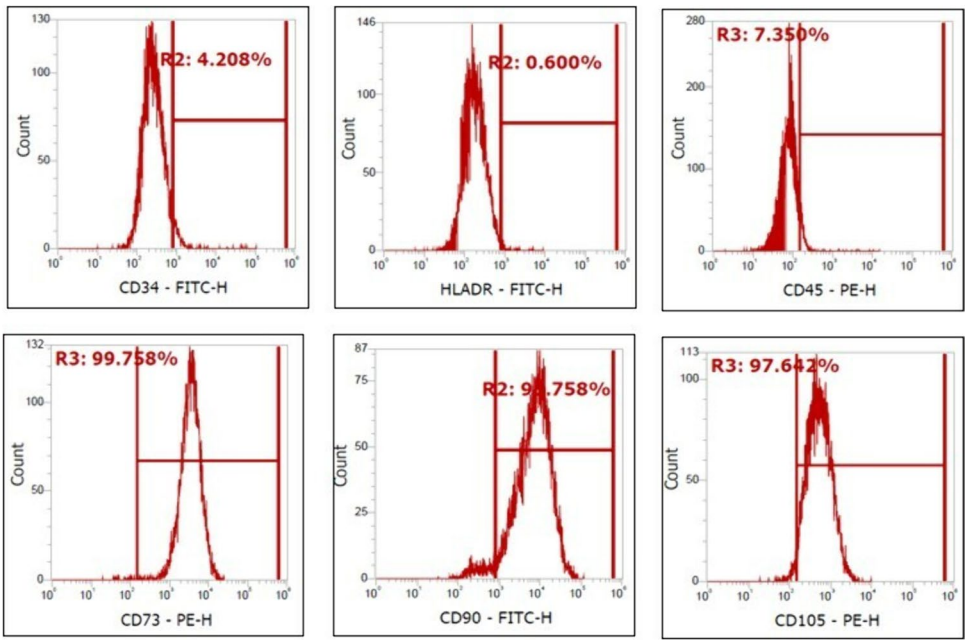


Figure 2. Representative FACS analyses show that DPSCs were positive for surface marker characterization of MSCs (CD105, CD73, CD90) and were negative for specific hematopoietic markers (CD45 and CD34).

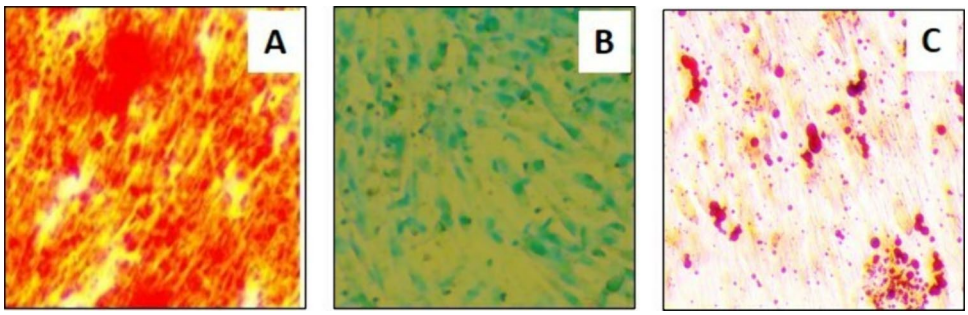


Figure 3. Trilineage differentiation of dental pulp stem cells. (*A*) Osteogenic, (*B*) chondrogenic, (*C*) adipogenic differentiation.

obtained from the previous treatment. Scaffold which was treated with 10% HCl was fragile (Fig. 4*D*). Scaffold treated with 1% HCL showed good physiological properties compared with 5% and 10% HCL (Figs. 4 and 5).

Cells were attached on the leaf surface and penetrated the leaf structure, mimicking their natural function. Cell growth was observed on the surface of the scaffold. DPSC cell growth was confirmed using confocal microscopy and scanning electron microscopy. The MSC cell colonies were observed on the scaffold (Figs. 6 and 7).

Osteogenic differentiation In scaffold A, calcium deposition is in higher number than that in scaffold B and positive control. The differentiated cells were stained by alizarin red dye to confirm calcium deposition. Calcium deposition was observed under a phase contrast microscope on a scaffold

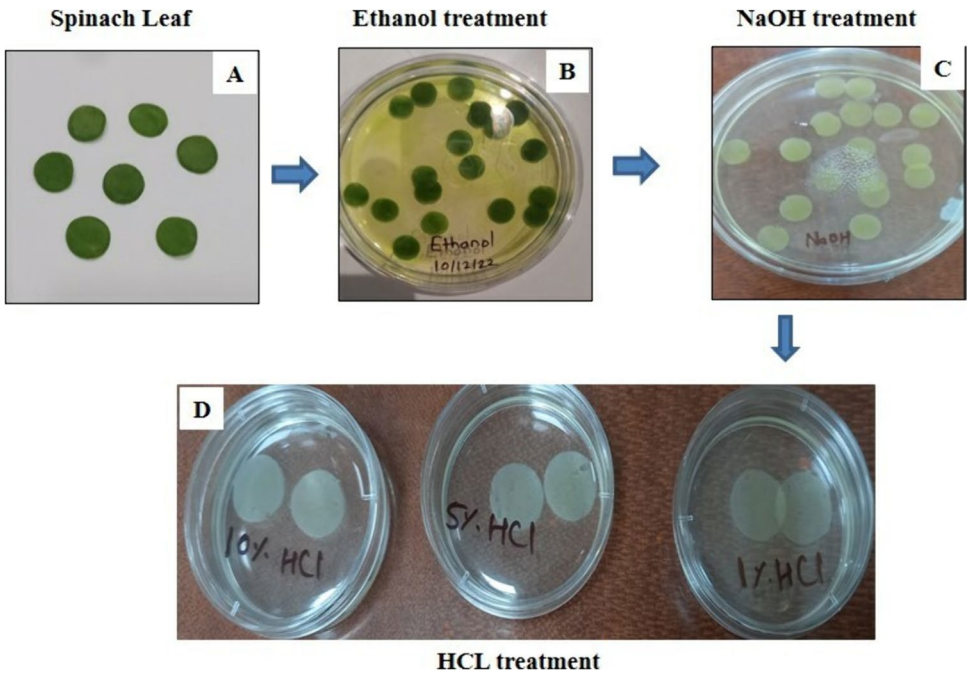


Figure 4. Preparation of scaffold by using serial chemical treatments.

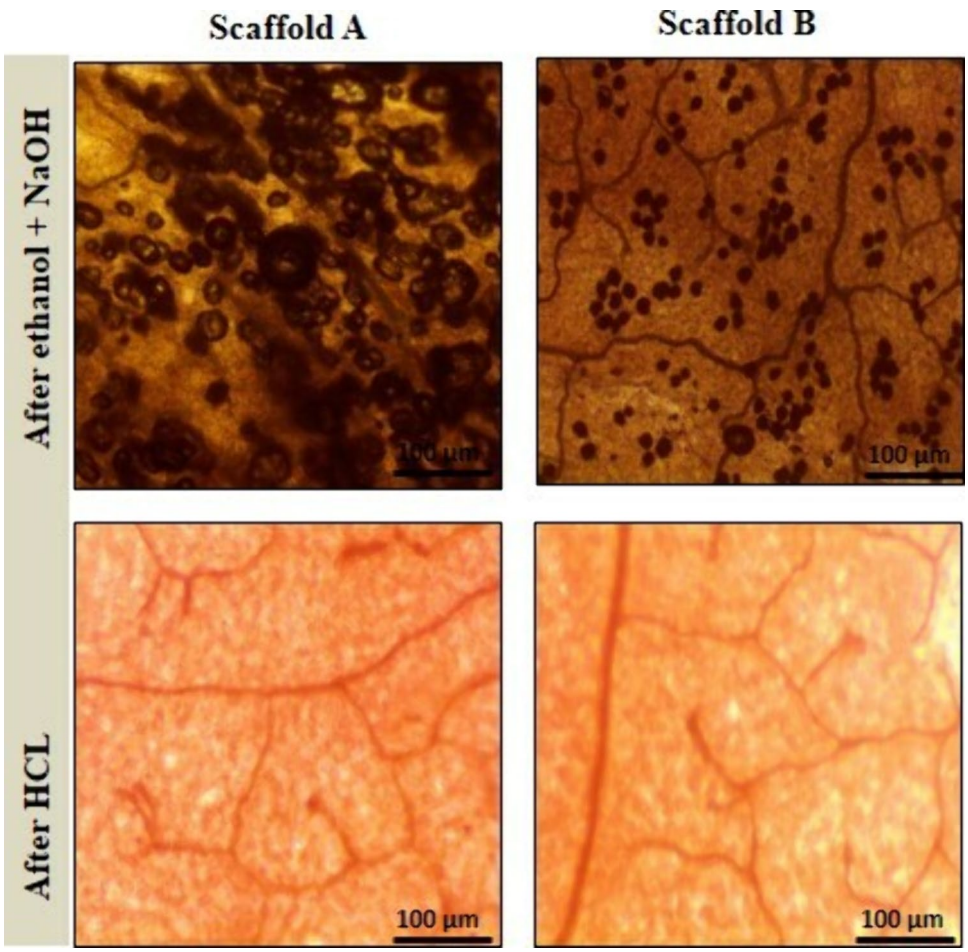


Figure 5. Structure of scaffold after treatment with ethanol, NaOH and HCL.

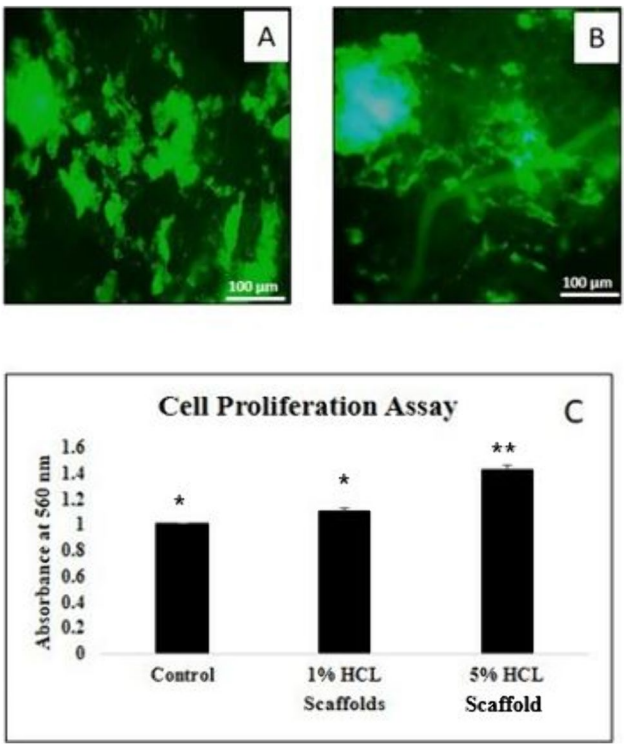


Figure 6. Confocal microscopy image of (*A*) scaffold A, (*B*) scaffold B and (*C*) cell proliferation by MTT assay; the data shown are the mean ± S.D., $n = 3$ vs. control: 1% HCL scaffold: 5% HCL scaffold.

(Fig. 8*A*). Further confirmation was done by measuring optical density at 450 nm by using 0.1% acetic acid (Fig. 8*B*).

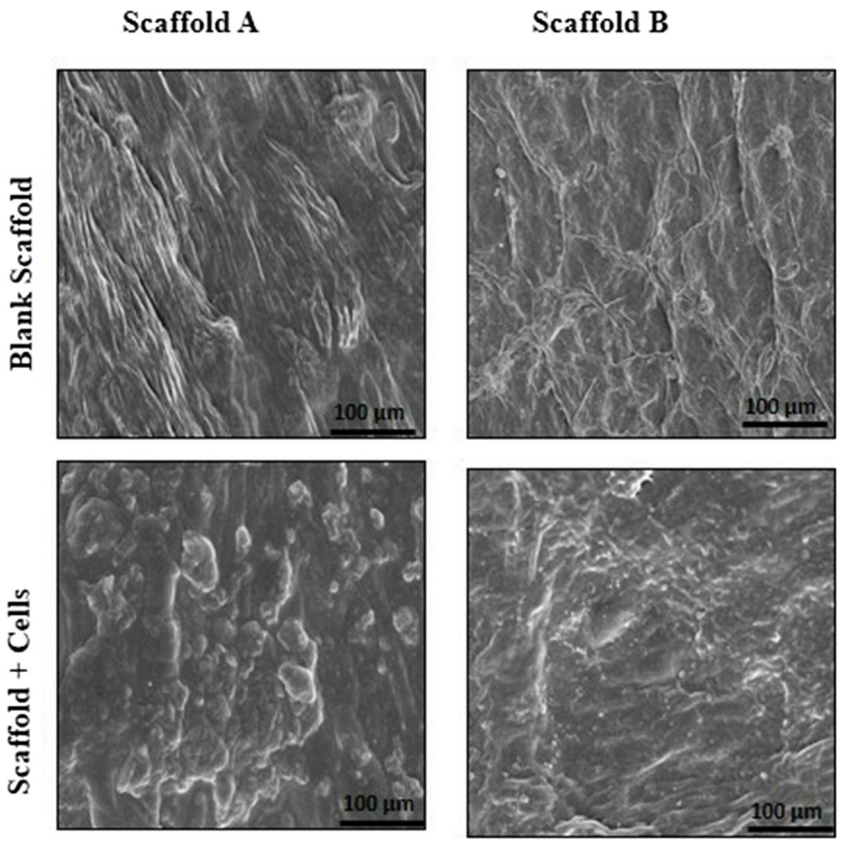
Gene expression Osteogenic gene expression was examined. Osteonectin (OSN) gene expression was shown in both control and plant-based base scaffold. At day 14, plant scaffold had significantly higher OSN than the control. In plant-based scaffold, osteonectin expressions increased by 2.5-fold compared with the control group, which is a statistically significant result (Fig. 9).

Discussion

Nowadays, availability of tissue donors and problems faced with tissue transplantation have become a necessity to grow cells with scaffolds, either natural or synthetic (Sharma *et al*. 2019). Scaffold is one of the most important three pillars of tissue engineering, inclusive of cell and growth factors which enable to form 3D structures (Hollister 2006).

Biological scaffolds are a valid alternative to traditional therapies and may improve outcomes or offer solution which may not be possible with synthetic scaffolds. Advanced research for different indications using various scaffolds has led to a thorough understanding of the mechanisms involved

Figure 7. Scanning electron microscopy image of scaffold A and scaffold B.



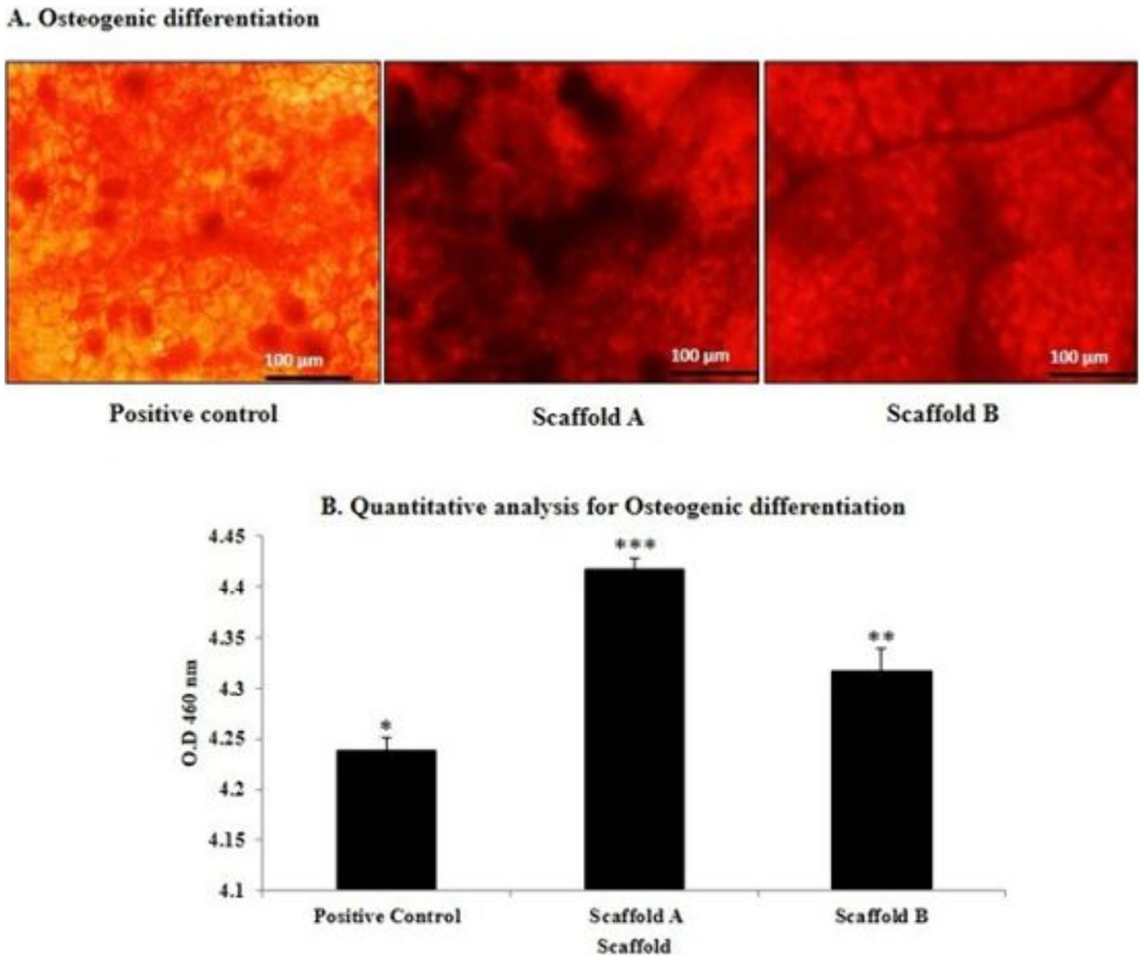


Figure 8. (*A*) Osteogenic differentiation stain by alizarin stain to confirmed calcium deposition. (*B*) Quantitative analysis: scaffold A showed significantly increased osteogenic differentiation as compared to scaffold B and positive control.

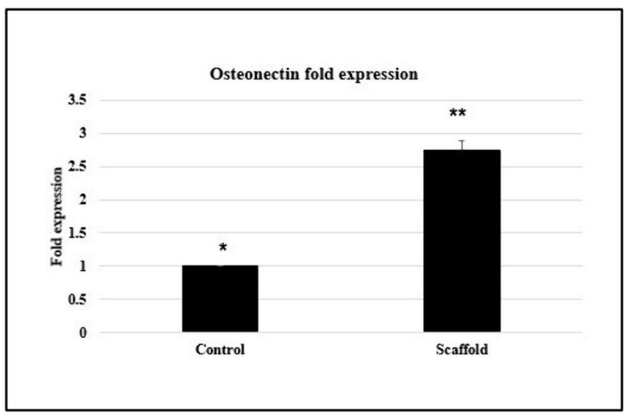


Figure 9. Osteogenic gene expressions measured by RT-qPCR. The mRNA levels of osteonectin (OSN) were measured from total RNA extracted from the control and plant-based scaffold after osteogenic induction. Data are expressed as mean ± SD; $n = 3$. GAPDH gene was used as a reference. The relative expression (fold increases) in plant scaffold compared to control.

in in vivo remodeling (Howard *et al*. 2008; Bružauskaitė *et al*. 2016; Lacombe *et al*. 2020).

Recently, plant-derived decellularized scaffolds are of a huge interest replacing animal sources for tissue regeneration (Harris *et al*. 2021). There is a built-in architecture in the plant leaves in the form of venation. These naturally designed decellularized scaffolds mimic the extracellular matrix of mammalian tissues due to suitable cytocompatibility (Contessi Negrini *et al*. 2020). Plant-derived scaffolds have been used for neural differentiation in regenerative medicine (Couvrette *et al*. 2023). Decellularized spinach leaf scaffold could be more successful in differentiating stem cell into the bone formation or bone repair. They allow the exchange of nutrition and oxygen and vascular ingrowth through the scaffold. It is providing a three-dimensional substrate by feeding and delivering oxygen to the cells. Stem cells can be guided to arrange in a specific direction depending upon the structure of the plant material used.

Plant-based scaffolds have a lot of practical benefits such as easy availability, mass production,

cost-effectiveness and ready to use for tissue engineering application. Plant leaves are made up of cellulose fiber—a non-toxic (Ilangovan *et al*. 2020). Large groups of cellulose molecules come together to form microfibrils. These properties make them suitable candidates for scaffolds in tissue engineering (Hickey *et al*. 2018). They compensate traditional scaffolds by providing a larger surface area, vascular networks, water transfer and retention properties. Our decellularized spinach leaf scaffolds were found to be non-toxic and enhance the DPSC proliferation (Fig. 6*C*). Earlier studies have demonstrated that plant-based scaffold of *Ficus religiosa* leaf skeleton architecture exhibited biocompatibility in mammalian cell adhesion, proliferation and functionality (Periasamy *et al*. 2020; Lacombe *et al*. 2020).

We used a sequential chemical treatment which is a gold standard approach for decellularizing plant tissue. Aqueous detergent (e.g. sodium dodecyl sulphate (SDS)) is traditionally followed by a surfactant-bleach solution (Adamski *et al*. 2018). In this study, we used sequential chemical treatment with NaOH, HCL and ethanol for the decellularization of spinach leaves. NaOH destructs the cell wall and removes chlorophyll while retaining the tissue structure. HCL helps in the removal of the cuticle (wax layer) and ethanol enables the sterilization of the leaf (Howell *et al*. 2022). In regenerative therapy, pore size is very crucial for a better growth of cells. Cells respond strongly to mechanical rigidity and flexibility, and 3D nano topology, as well as extracellular stimuli (Calin and Paun 2022). Our data shows that 5% HCL–treated spinach leaf scaffolds support attachment and proliferation DPSCs compared with 1% HCL. Further, spinach leaf scaffolds must be sterilized before introduction to cell culture environments. Plant-based scaffold does not show toxicity in DPSCs. UV radiation and ethanol methods have been previously employed (Dai *et al*. 2016). We used spinach leaf scaffold for assessment of the osteogenic differentiation potential of human DPSCs. These decellularized scaffolds enhanced the osteogenic differentiation in terms of calcium deposition and bone-related gene expression such as osteonectin (OSN) (Dhandayuthapani *et al*. 2011).

In the bone, osteonectin (OSN) is a glycoprotein secreted by osteoblasts that binds to calcium while the bone is being formed. This initiates the mineralization process and encourages the production of mineral crystals. OSN is a noncollagenous extracellular matrix protein and has been suggested to bind selectively to both hydroxyapatite and collagen, and to link the bone mineral and collagen phases, which is likely to lead to active mineralization in normal bone tissue.

The results of the scanning electron microscopy analysis showed that the spinach scaffold in NaOH with 5% HCl provides ideal surface for trapping cells. The decellularized spinach leaf scaffold exhibits better cell attachment, growth, proliferation and differentiation. The plant scaffold could thus be considered an alternative for synthetic scaffolds in tissue engineering (Fontana 2019).

In this investigation, we proposed unconventional alternative tissue engineering plant-based scaffolds for bone regeneration. The spinach leaf scaffolds are promising alternative biomaterials for bone tissue engineering and improving their regenerative abilities for repairing damaged bone. The unique features of the plant scaffolds can offer inner vasculature, optimal fluid transport, etc. to make it an alternative natural model. However, they have few limitations such as high hydrophobicity, absorption of small hydrophobic molecules and non-biodegradability. This finding provides the potential of decellularized leaf scaffold and DPSCs as a therapeutic regenerative medicine for treating bone defect, which could be of great academic and clinical significance. More research needs to be carried out to explore this field as an ideal source for tissue engineering.

Statistical analysis The ANOVA test was followed by Scheffe's multiple comparison test to compare in vitro analysis. All results are presented as mean ± SEM or mean ± SD. The mean difference is significant at the 0.05 level.

Conclusion

Our data demonstrate that the decellularized spinach cellulose scaffolds stimulate the growth, proliferation and osteoblastic differentiation of DPSCs in addition to providing a 3D culture environment. Our study represents a case of symbiotic relationship between plant cellulose scaffold and human stem cells.

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Author contribution Kautubh Raundal and Dr. Avinash Kharat planned and performed experiments and wrote the first draft of the manuscript. Pranjali Potdar and Swapanli Sakhare, gene expression study. Dr. Ramesh Bhonde supervised the work. Dr. Avinash Sanap and Dr. Supriya Kheur edited the manuscript. All authors have read and approved the final version of the manuscript.

Data availability Data is available from the authors upon request.

Code availability Not applicable.

Declarations

Ethical approval The study is approved by the Institutional Committee for Stem cell Research (IC-SCR) no. IC-SCR/001/2022 Dr. D. Y. Patil dental college and Hospital, Pimri Pune.

Consent for publication All authors agreed to the publication in the submitted form.

Competing interests The authors declare no competing interests.

Declaration of AI All authors did not use AI tools to analyse and draw insights from data as part of the research process.

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