Int. j. adv. multidisc. res. stud. 2024; 4(2):751-756

Received: 08-02-2024
Accepted: 18-03-2024

International Journal of Advanced Multidisciplinary Research and Studies

ISSN: 2583-049X

Study on the Lupine-hulls Based Cellular Scaffolds for Human Cardiac Tissue Regeneration

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DOI: https://doi.org/10.62225/2583049X.2024.4.2.2572
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Abstract
It's becoming more popular to develop new biomaterials enabling *in vitro* cell cultivation with microbial environments of biological structures. The novel strategies for creating native cellular environments that facilitate the growth of human cardiac progenitor cells (hCPCs) (Sca-1^{pos}) and scaffolding more closely resemble authentic cell function microenvironments with offer a wide range of appealing *in vivo* and *in vitro* uses. Both the biologically active substances and the mechanical properties of the hull scaffold were studied. SEM analysis of the hull's microstructure reveals that the inside surface shows its large and naturalistic activity, while the outside surface displays the celular-structures. Tensile properties, high-performance liquid chromatography (HPLC), and fourier transform infrared spectroscopy (FTIR) spectra are further characteristics of the lupine-hulls. Scaffolds made from lupine-hulls are cheap, simple to make, and from a renewable material. The MTT assay for cell proliferation served to evaluate the impact of the hulls that are solubility substance on the Sca-1^{pos}. The cell lines utilized in this study were able to multiply and maintain high viability even after being cultured for 7 straight days. Results also demonstrate that the nuclei of the cells were visible under fluorescence microscopy and remained viable within the cellular structures of the hulls *in vitro*. This research showed that scaffold made from lupine hulls are being used as biological scaffolds for *in vitro* cell growth, which may play a role in the tissue engineering of hCPCs.

Keywords: Vegetable-Waste Biomaterial, Sca-1^{pos} Cell-Culture, Lupine-Hull-Cellular, Cellulose Scaffold, Cell-Proliferation Assay, Lupine-Hull Property

1. Introduction

Even though allogeneic tissues and organs have been clinically transplanted successfully multiple times to address disorders brought on by malfunctioning or defective organs or tissues, there are currently none of those therapies available due to a lack in allogeneic donors and health issues [1]. Tissue engineering represents a novel discipline in bioengineering that combines materials science, biology of cells, and design to construct tissue replacements that mimic the anatomical and physiological properties of natural tissues [2]. Tissue repair by tissue engineering may be able to solve organ scarcity in the clinical emerging [1]. Several studies exist for utilizing organic scaffolds to facilitate cell proliferation and *in vitro* growth [3-5]. The fact that cellulose and its derivatives cause a somewhat strong foreign body reaction in tissue and generally tolerated well by tissues and cells is the driving force motivating this advancement [6]. An innovative field of study towards the reusing and recycling of garbage as well as the generation of novel, sustainability biomaterials for uses in medicine can both benefit from the use particular organic wastes as a low-cost source for useful agricultural products [5-7]. Using innovative biocompatibility structures, the physiological milieu of the extracellular matrix (ECM) might be replicated in every aspect of its complexities, including its biochemical and physical characteristics [8]. Fruits were successfully utilized to produce naturally compatible and a cellulose- scaffolding which was implantation for 3D mammalian cell cultures [3-4, 9]. Additionally, cellulose-porous structures were used to encourage significant osteoblastic development of an induced pluripotent stem cells (iPSCs) as well as human mesenchymal stem cells, taking advantage of clinical possibilities for these cells' usage for *in vivo* implantation [10-12]. Mammalian endothelial, cellulose-porous structures were seeded with mesenchymal and pluripotent stem cells that are maintaining their practical features [13]. Hence, while a gradual non-enzymatic β(1-4)-bond hydrolysis drives cellulosic breakdown in tissues, and cellulose can be considered as a very stable matrix.

In this study we used a simple method to produced cellular structures of lupin-hulls by washed with bi-distill water and then lyophilization dry. The hulls are then characterized by tensile strength, cellular microstructural by SEM, Fourier transform infrared spectroscopy (FTIR) spectra, High performance liquid chromatographic (HPLC). We have investigated bioactive compounds and various characterized properties of the hulls and the protective effect toxicity in cultured with Sca-1^{pos} cells proliferated within these cellular-structures for up to 7 days. These demonstrated ability to multiply and maintain viability within cellular-structures *in vitro*, attaining cell numbers comparable to those of other synthetic and natural biomaterials. Because of their inherent porosity and adaptability to change their mechanical characteristics, scaffold with cellular structures, we show that a cellulose- scaffolding are a biomaterial with prospective applications that may be effectively used for *in vitro* cell growth.

2. Materials and Experimental

2.1 Materials

Human Cardiac Progenitor Cells (Sca-1^{pos}) are presented from Department of Internal Medicine, Laboratory of Molecular and Cellular Cardiology (University of Rome Tor Vergata, Rome, Italy). The Sca-1^{pos} were kept in Dulbecco's Modified Eagle Medium (DMEM), which was enhanced with 10% FBS and antibiotics (streptomycin and penicillin). The cells are incubated at 37°C in a humidified 5% CO_2 environment.

Following their processing to remove the kernel for food and any leftover hulls, the lupine hulls were acquired from the nearby provider in Rome, Italy. The hulls were washed several times in double-distill water, followed by Lyophilization dry for about over a night before the characterizations.

Hulls were placed in ethanol with magnetic stirrers in a full day at 23°C. following this, they were repeatedly rinsed with double-distill water to get rid of any remaining traces of ethanol. After that, they were lyophilized and kept at room temperature (23°C) with a dry place.

Hulls were placed in ethanol with magnetic stirring over a whole day at 23°C. Following this, they were repeatedly rinsed with H_2Odd to get rid of any traces of ethanol. They were then lyophilized and kept at room temperature under dry conditions. The hulls were cut into disk with a centimeter of size and rehydrated using phosphate buffer solution (PBS).

2.2 Lupine-hulls Characterization

The hull morphology was observed by means of scanning electron microscopy (SEM) (Cambridge Stere-oscan 300, Leo Supra 35). Qualitative observations of hulls surface morphology were carrying out using FE-SEM model Ziess Supra 55VP. The hulls samples were fixed to an aluminum stub and then coating with gold in a BIO-RAD-Polaron-Division-SEM coating system to minimize electron charging effects during examination.

The tensile strength and Young's modulus of the hull have been measured using the tensile test technique (ASTM-D3822-01). Hulls were selected at random, attached, and adhered to a paper tab prior to measurements. Uniaxial tensile tests were used for the testing, possibly with a sample gage length of 30 mm and a crosshead rate of 5 mm/min. The hull specimens were trimmed to size and sandwiched between two cardboard sheets that were 30 mm wide by 70 mm long. The cardboard example is displayed in Fig 1, where 20 millimeters hole was made in the middle of the cardboard. The support cardboard's edge was trimmed to remove loads from the frame before testing. Five samples of lupine-hull specimens were examined in this study.

The Fourier transform infrared (FTIR) spectrometer (Jasco, FT/-6600) was used to capture IR spectra at a resolution of 4 cm^{-1} during the 4000 cm^{-1} range. The hull specimen was used for the investigation. Attenuated total reflection cells were used to perform the IR measurement, with a resolution of 2 cm^{-1} across 32 scans.

A Shimadzu chromatograph fitted with two LC-10AT Shimadzu pumps, an SPD-10Ai UV-Vi s detection device, and a Pinnacle II C18, 5 µm, 10×4.6 mm column stored in a Shimadzu CTO-10A oven with a temperature setting of 30°C was used to execute high performance liquid chromatography (HPLC). At a flow rate of 0.8 and 0.6 ml / minute, the phase in motion consisted of 92.5% methanol and 7.5% water. Materials are carefully fed into a 200 µl container, and data caotured using LC Solution software (version 1.25; Shimadzu Corporation: Kyoto, Japan, 2002–2009) with a wavelength of 210 nm.

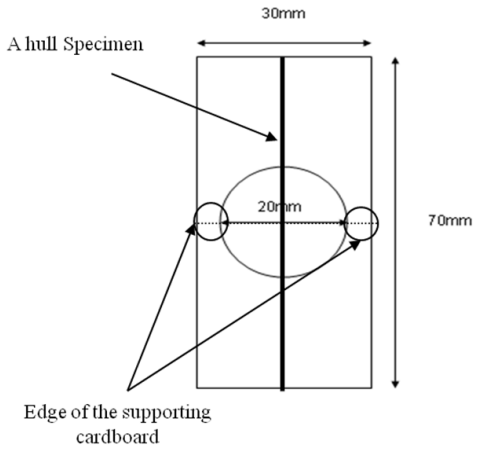

Fig 1: Illustration of paper which used for tensile tests of lupine hull

2.3 Cells culture studies

2.3.1 MTT Proliferation Assay

The colorimetric MTT test has been utilized to determine the development of cells. Initially, the hCPCs Sca-1pos cell line was kept at 37°C in an incubation chamber and 5% CO_2 mixed DMEM (Gibco) with 10% FBS (Gibco). Trypsin/EDTA was used for collecting monolayer Sca-1^{pos} cells. Hull scaffolds were UV-sterilized prior to introducing cells. The hull scaffolds were pre-soaked in DMEM for a minimum of 12 hours following sterilization. After that, the scaffolding elements were cultured between 3 and 7 days at 37°C in an incubation chamber having 5% CO_2 using 24 well tissue culture plates with Sca-1^{pos} cells at a density of 2 × 105 cell/mL. Following incubation, the cell-scaffold constructions were washed with PBS to exclude non-adhering cells, and then incubated over four hours with 50 µL MTT reagent at the previously mentioned conditions. DMSO 500 µL was applied to each well to dissolve the converted dye once the media had been eliminated. A 96-well plate is used for holding the solution (150 µL) for all samples, while a reader of an ELISA used to determine the optical density (O.D.) at 490 nm absorbance. Utilizing the

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) test, the impact of the hull on the viability of Sca-1^{pos} cells was assessed. Added another 700µl in 24-well microtiter plates once 1.4 x 104 cells (100µl each well) had been distributed in the hull with 200µl of complete culture media. After incubation for 3 days and 7 days at 37°C viability of the cells during 3 and 7 days of culture at 37°C using a humidified incubator.

2.3.2 Fluorescence Microscopy

Optic microscopes are known as fluorescence microscopes which use the fluorescence phenomena to investigate the characteristics of organic or inorganic materials. In this study, the cell-seeded constructs were prepared as described above (cell concentration $3 \cdot 10^6$ cells/ml) and fluorescently stained for visualization with the confocal microscope. First, fixation of the cells was performed with 3.7% Solution of formaldehyde into PBS about 10min at 23˚C. This process has purpose of preserving tissues from decay and maintaining the cellular structure. Formaldehyde is a cross-linking agent which creates covalent bonds between the proteins of the cell, fixing them to the cytoskeleton and preventing the proteins from shape changes due to denaturation. The samples washed 3 times for 5-10 mins in PBS. Permeabilization of the membrane as carried out using PBS containing 0.1% Triton X-100 solution, which allows the staining solutions to penetrate the membrane and reach the labeling sites. The samples are again washed 3 times for 5-10 mins in PBS. The staining solution was prepared diluting 5µl of phalloidin stock solution 6µM in methanol in 200µl of PBS, with final concentration 0.15µM. The hulls were stained for 1h at 23˚C. The stains solution was made of 0.7µl 5mM stock solution in DMSO in 1ml PBS, with final concentration 3.5mM; the staining was performed for 10 mins at 23˚C. The samples are eventually washed 4 times in 10 mins with PBS to eliminate any of the unbound staining, and placed on a cover slip for visualization, or either kept at 4˚C.

3. Results and Discussion

3.1 Morphology of the hulls.

The hulls are yellow, sometimes with patches of dark brown color. Previous research on hulls revealed that between 26.6% and 36% of the total seed mass is fresh. When hulls thickness was examined by species, it meant roughly 400 µm (330 µm – 530 µm) [10]. Naturally occurring polymers like lignin and also pectin to bind a group of each cell together to form multicellular naturally cellulose yarns. Fig 2 showed SEM micrograph of the hull surfaces of inner and outer. Comprising depressions of varying fiber diameters on the macrosclereid layer, variable layouts of cuticle fractures, and deposits of cuticle parts as shown in Fig 2(a), the outer surface displayed significantly of variety in seeds sculpting. The fiber's internal structure is shown in Fig 2(b), which shows that each biofiber single cell has a in lumen, an open chamber, and a porous framework. Its hollow lumens serve as an acoustical and heat insulator with reducing the bulky volume for the fibers. The dehydrated hulls samples showed a crushed internal cell structure. This decreased the amount of vacuum in the pores because dry surface broke down the cell walls of the hulls. The organic micro-porosity which was detected indicated the hull scaffolding might be semi-permeable, which would also permit gases exchange. Furthermore, despite the hull's preparations procedure causing decreases in comparison with the raw hull, its antimicrobial capacity remained intact, suggesting that the anti-bacterial phytochemicals are may potentially be released.

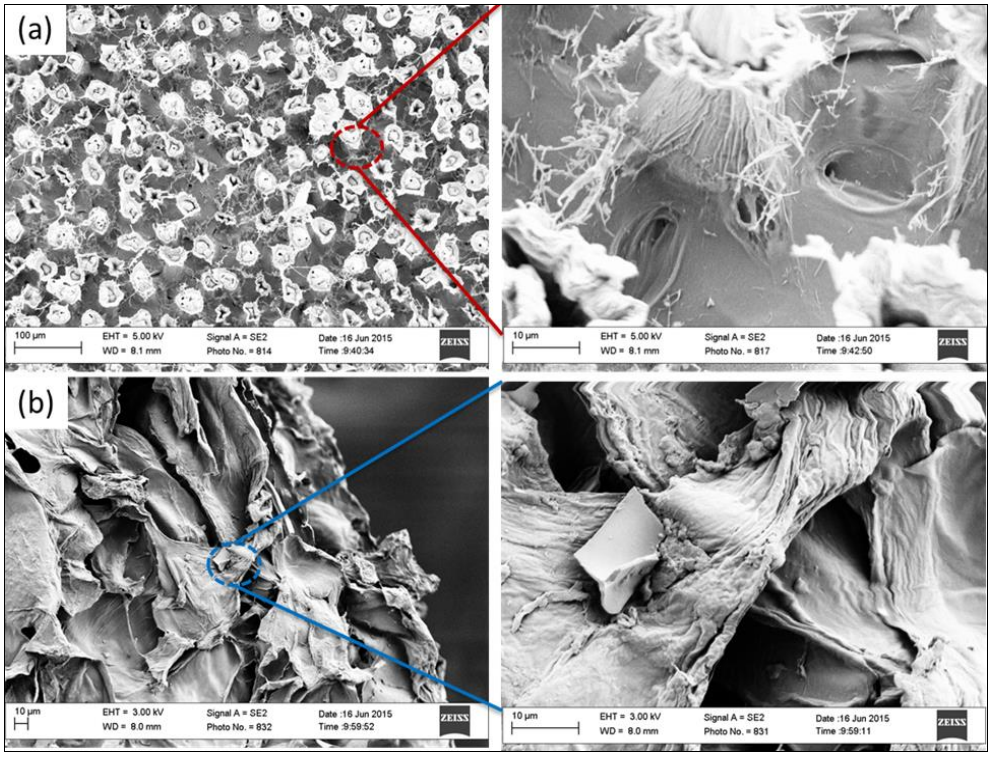

Fig 2: SEM Morphology of lupine-hulls (a) outer surface (b) inner surface.

3.2 Tensile properties

Fig 3 displays the stress-strain and strength and modulus of hulls specimens with and without lyophilization dried in the wed and dried condition. As expected, all wed samples exhibited significantly lower tensile properties than dried samples showed in Fig 3(b) & 1(c). Hulls with lyophilization dried had slightly lower tensile strength and strain than natural dried and natural dried samples which also indicate lower tensile modulus than sample with lyophilization dried. However, the differences in tensile strength and modulus between natural dried and lyophilization were not significant, suggestive of a greater sensitivity of strength to macropores than of modulus and strain. The notably reduced strain, modulus, and strength of wed sample was simply due to its cellular structure absorbed water and therefore, cell walls of hulls stand (more pore size) when compared to additional dried specimens suggesting that the tensile properties of hull cell specimens were not limited by pores size; pores volume was also an important factor. Regarding modulus and fracture stress, the hulls exhibited a fragility characteristic. The potential for developing and manufacturing biological materials alongside tailored mechanical attributes to address particular use requires across a range of industries, in packaging to medical science, is suggested by these findings as well as those of prior research utilizing resources made from plant-based waste [14-17]. By changing the type of plant-based, compounds with various mechanically, chemically & physically characteristics can be obtained.

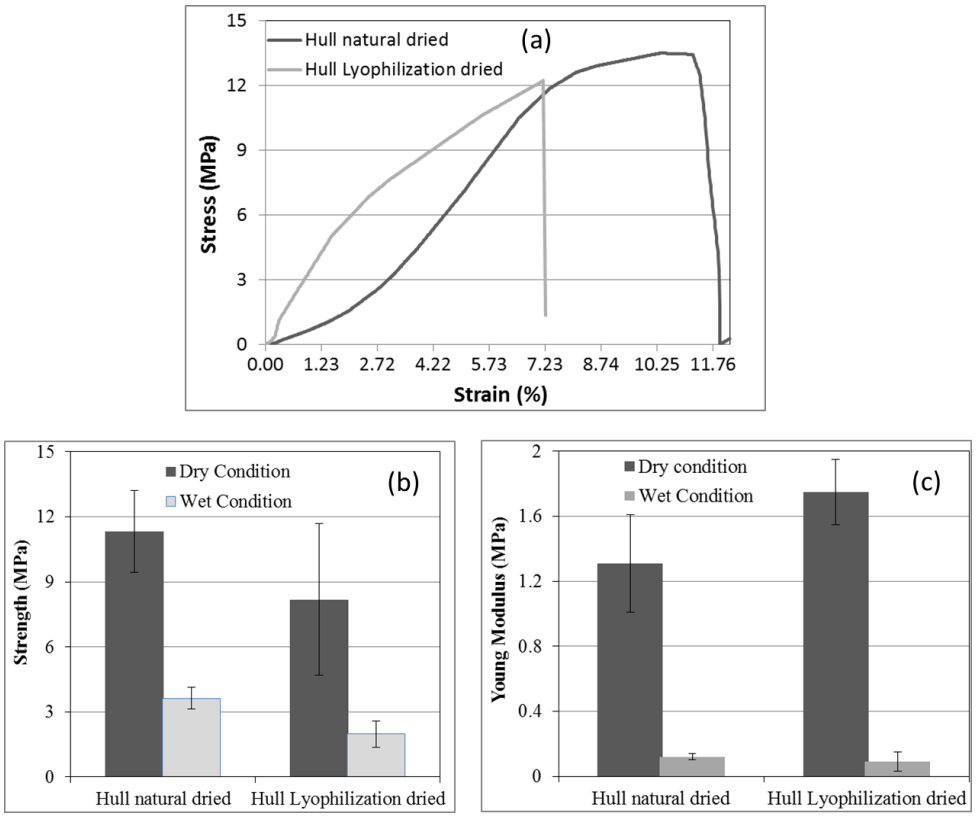

Fig 3: Tensile properties of lupine-hulls with and without lyophilization dried and wed condition: (a) Stress-strain curves, (b) strength, and (c) modulus

3.3 FTIR analysis

FTIR spectrum of hydroxyl group, Both the O-H bond vibration and the C-O group showed extremely wide bands at 3384 cm^{-1} and 1033 cm^{-1}, respectively, with moderately strong bands at 764 cm^{-1} and 1033 cm^{-1}. The unsaturated part's out-of-plane C-H vibration was detected at 2916 cm^{-1}. The equivalent of C=C vibrations were observed in a faintly strong range at 1654 cm^{-1}. Using the strength band around 2916 cm^{-1} and the medium's strength range of 1460 cm^{-1}, both stretching and bending motions of the methyl portion were observed. The range around 2851 cm^{-1} and the middle range around 1548 cm^{-1} indicated the methylenic portion's vibrations. It was determined that the shaking action of methylenic par played a role for the moderately powerful spectrum around 764 cm^{-1}. The equivalent of C-C vibrations appears around 1033 cm^{-1} in a small region. The common cellulosic pattern was shown using FTIR spectroscopy examination in the lupine-hull molecular spectra, which was similar to its chemical makeup of the utilized plant-based waste.

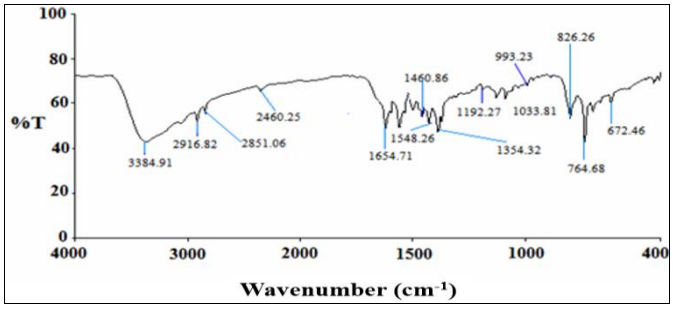

Fig 4: Displays the FTIR spectrum of Lupine-hull carried out using these specifications: 32 scans, a spectral accuracy at 2 cm^{-1}, and a wavenumber spectrum at 400–4000 cm^{-1}

3.4 High performance liquid chromatographic (HPLC)

In this experiment the protocol for extracting compound were used by assessing the performance in HPLC analysis of lupine hulls-soluble fraction in hexane and ethyl acetate, respectively. The hulls used to perform in HPLC analysis were no Lyophilization dried. The both solvents extract of the hulls contained the high concentrations of lupeol is shown in Fig 5. There seems to be a mechanism of resistance towards glutamate as well amyloid-β toxicity based on lupeol and β-sitosterol concentration. With respect to safeguarding Sca-1^{pos} tissues, the hulls extract containing hexane and ethyl acetate demonstrated the highest quantities of all the chemicals examined. A pentacyclic triterpenoid, lupane, contains a group called hydroxy substituting for a hydrogen bond at spot 3β, corresponding to the Chemical Entities of Biological Interest (ChEBI) [18]. It can be found in the latex from rubbery trees, fig plants, and lupine seed cover. Additionally, a lot of eatable fruit and vegetable products contain it.

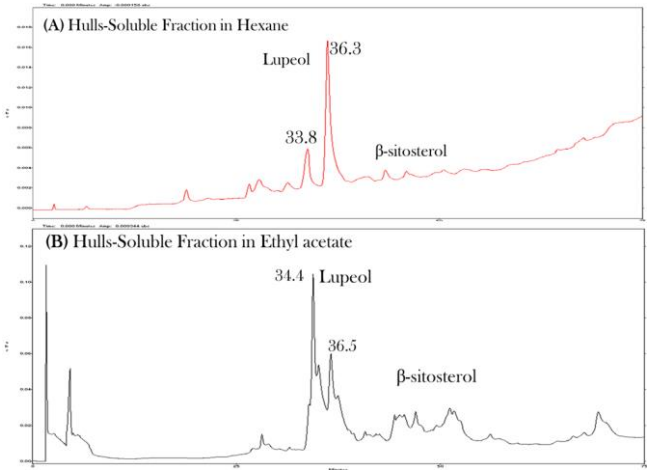

Fig 5: Chromatographs of lupine-hull extracts, (A) Hexane extract of lupine-hulls and (B) Ethyl acetate extract of lupine hulls

3.5 Cell Viability and Proliferation

The MTT assay finding displayed by Fig 6(a-b) showed that hCPCs-Sca-1^{pos} cells remained viable while their respective cell lines continued to proliferate at a steady rate for 3 and 7 days respectively throughout their cultures. The variation in cell growth revealed that the dimension and pores of the tumors engineering scaffolding are essential for the Sca-1^{pos} cultivating cells.

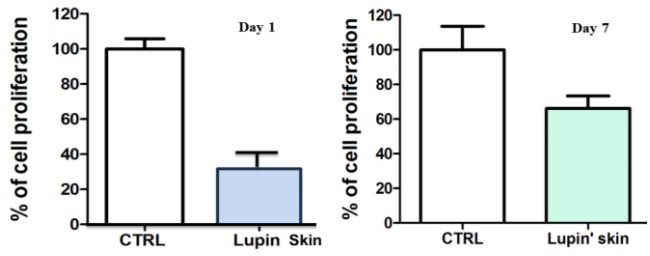

Fig 6: Proliferation of hCPCs, Sca-1^{pos} cell lines are cultivated with a hull cellular-structure via MTT Assay for 3 days, and 7 days

3.6 Fluorescent Microscopy with Confocal Imaging

Fig 7 shows significant circumferential labeling of the junctional proteins occluding indicated the existence regarding tight connections. Immunofluorescence staining showed that hCPCs, Sca-1^{pos} expressed cell nuclei on hull scaffold. To substantiate the positive impact of bioactive compound presented in hull scaffold on Sca-1^{pos} cells, we observed how nuclei were shaped subsequently to Hoechst 33342 stains. According to Fig 6 (enlarged confocal microscopic), for both patterns, which is red and blue microscopy, Sca-1^{pos} cells had regular nuclei, caused the chromatin condensation and nuclear contraction (shown by white arrows). This *in vitro* research proved the biological compatibility of the hulls scaffolding especially its prospective application as a structure for the proliferation of stem cells. It has been shown that according to this instance of hCPCs Sca-1^{pos}, hull has the ability to stimulate adherence of cells, development of cell interactions, and growth. Immunofluorescence tests demonstrated a significant proportion of viable nuclei in cells and elevated α-SMA protein production, indicating the scaffold's potential to trigger the hMSCs development technique's first dedication. Subsequent investigations into how genes behave will enable research to confirm the presence of α-SMA with additional proteins implicated in the mechanism of muscle development. For *in vitro* 3D cultivation of engineering tissues, a number of plant-based scaffolding are being used recently [19-21]. MC3T3-E1 pre-osteoblasts were cultured on a decellularized apple scaffolding, exhibiting strong attachment, growth, and osteoblastic differentiation [12]. Subsequently thus is feasible to use an organic substance derived of wastes having anti-microbial properties to construct a stem cell-scaffold framework, as evidenced by the capacity to sustain human stem cells' longevity, growth, and cell-to-cell connection that has been shown herein.

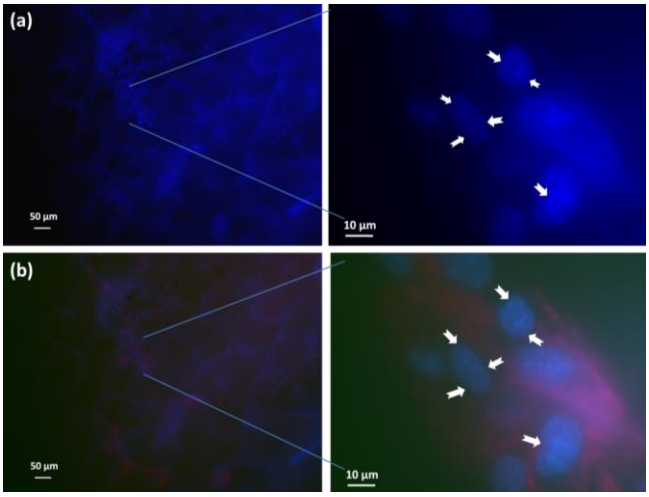

Fig 7: Nucleus of Sca-1^{pos} cells seeding on the cellular-structures of hulls by fluorescent microscopy. Phase-contrast fluorescence microscopy used to identify the Sca-1^{pos} cells subsequent to Hochest 33342 stains; white arrows indicate Sca-1^{pos} cells, (a) Blue pattern and (b) Red pattern

4. Conclusion

In the present study, hull-scaffolds raise concerns regarding application *in vivo*. The bioactive compound lupeol is found in lupine seed-coats. The lupine hull extracts' hexane and ethyl acetate exhibit significant free radical scavenging properties, further shielding Sca-1^{pos} cells from amyloid-β with glutamate damage. The hulls hexane extract contains a reasonable amount of all the chemicals measured by FTIR and HPLC, suggesting that β-sitosterol with luteol may be more important in protecting Sca-1^{pos} cells toward glutamate, while their ability to guard against amyloid-β

toxicity is still unknown. To sum up, the lupine hull has been recognized as a renewable scaffold due to its inherent porosity in cellular structure scaffolds with several chemicals that could have pharmacological activity, additional investigation on this and we demonstrate that hull scaffolds are potential uses as biomaterials that are effective for *in vitro* cell cultivation.

5. Acknowledgements
The author would like to express appreciation for the support of Department of Mechanical Engineering, National University of Laos and University of Rome Tor Vergata.

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