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# Morphology of medicinal plant leaves for their functional vascularity: A novel approach for tissue engineering applications

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**Abstract**
Despite significant advances in the fabrication of engineered biomaterials for regenerative medicine, delivery of nutrients in complex engineered human tissues remains a challenge. By taking advantage of the similarities in the vascular structure of plant and animal tissues, an attempt was made to develop prevascularized scaffolds from plant leaf for biomedical applications. The present study was undertaken on different medicinal plant leaves (n=6) to evaluate the morphology and functional vascularity before and after decellularization process. The strength of the leaves was assessed subjectively and it was observed that handling strength was apparently better in all the plant leaves studied except in betel leaves. The vascular patency test was done after decellularization and it was observed that the patency was retained even after decellularization process. Then the leaves were subjected for histological processing to assess the efficiency of decellularization process. Histological staining showed the loss of nuclei from the decellularized leaf without affecting the normal structure of the vasculature/tissue, as noted when leaves were stained before and after decellularization.

**Keywords:** morphology, medicinal plant leaves, decellularization and functional vascularity

## Introduction
The autologous, synthetic and animal-derived grafts currently used as scaffolds for tissue replacement have limitations due to low availability, poor biocompatibility, and cost. Plant tissue have favorable characteristics that make them uniquely suited for use as scaffolds, such as high surface area, excellent water transport and retention, interconnected porosity, preexisting vascular networks and a wide range of mechanical properties (Adamski *et al.*, 2018) [2].

The biochemical and physical properties of extracellular matrix (ECM) have a significant impact on numerous critical physiological and developmental processes (Griffith and Swartz, 2006) [8]. Significant morphological and biological differences have already been observed between cells grown on 2D versus 3D microenvironments (Pontes *et al.*, 2012) [11]. It is has been routinely observed that primary cells isolated from tissues will become progressively flatten when cultured on conventional 2D surfaces (Petersen *et al.*, 1992) [12].

Conversely, cells cultured on 2D surfaces can regain their 3D morphologies when placed into a 3D culture scaffold (Benya and Shaffer, 1982) [3]. 3D cell culture promises to more closely reflect the biochemical and physical properties of the cellular microenvironment found in tissues and organs (Owen and Shoichet 2010) [13] and so the development of novel biomaterials towards this effort is of considerable importance.

Both synthetic and naturally derived materials are currently employed in 3D culture methods, in order to create tunable scaffolds engineered with specific biochemical and physical properties. Cellulose, the major component of plant cell walls, is an organic polysaccharide made of D-glucose subunits through β (1–4) bonds. Unlike the polysaccharides starch and glycogen, cellulose provides very little nutritional energy as the β (1–4) glycosidic bonds are difficult to digest and can only be broken down by cellulose. As such, there has been a great focus on using cellulose as a candidate biomaterial and synthetically produced cellulose scaffolds have also been employed for 3D mammalian cell culture (Derda *et al.* 2011) [6]. These suggest that cellulose may be a suitable material to support 3D cell growth.

Moreover, cellulose is widely available as it is the most common organic polymer, accounting for 1.5 X $10^{12}$ tons of total annual biomass production (Klemm *et al.* 2005) [10].
These naturally developed characteristics are important in any scaffold employed for 3D cell culture (Bancroft *et al.* 2003) [4]. In order to act as a 3D scaffold, the medicinal plant leaf tissue must first be decellularized in order to remove existing nucleic acids, lipids, and proteins, producing a purified cellulose scaffold. Decellularization is now commonly employed on mammalian tissues to selectively remove cellular components while leaving behind an intact ECM (Bourgine *et al.* 2013) [5]. Typically, mammalian tissues are bathed in solutions, detergents and/or proteases, in order to produce a decellularized matrix that retains the shape of the original tissue. Decellularized tissue can then be repopulated with new cells, in order to produce new functional organs. Hearts, kidneys have been decellularized and reseeded with various cells. As well, functional bladders and lungs have been produced and transplanted into animals using this technique (Atala *et al.* 2006) [1]. Importantly, decellularized tissue also maintains a well conserved native ECM architecture and cell-ECM binding domains (Ott *et al.* 2008) [14].
In this study, we hypothesized that decellularized plant leaf tissues might provide an easily produced scaffold for 3D cell culture and biomedical applications. The major aim of this experiment was to study the macroscopic and microscopic structure of decellularized plant leaf tissues for development of 3D cellulose scaffold *in vitro*. Through modification of an existing decellularization protocol, we prepared and compared the cellulose scaffolds of various plant leaves for cell culture applications. Light microscopy and patency tests were used to characterize the structure of the vascularized scaffolds. We concluded that the leaves of *Sauropus androgynus* used in this study were able to produce ideal scaffolds similar to other synthetic and natural biomaterials. Given the natural porosity and ease of production of cellulose scaffolds, as well as the ability to modify their mechanical properties, we have attempted to demonstrate that cellulose scaffolds are a potentially useful biomaterial that can be successfully employed for *in vitro* 3D cell culture applications.

## Materials and Methods

The leaf materials for the present study were collected from different medicinal plants from Veterinary College, Shivamogga. We collected fresh leaves and immediately immersed in the distilled water for 24 hours. The sequence of treatments for pre and post decellularization steps were designed and depicted in Fig 1 and Table 1. After soaking in the distilled water, the leaf samples were transferred to freezer and thawed twice.

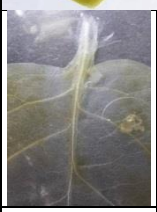
| S. No | Processing sequence | *Piper betle* | *Sauropus androgynus* | *Basella alba* | *Azadirachta indica* | *Centella asiatica* | *Mentha spicata* |
|---|---|---|---|---|---|---|---|
| 1. | Fresh Leaves |  |  |  |  |  |  |
| 2. | freeze thaw cycle |  |  |  |  |  |  |
| 3. | Dechlorophyllazation |  |  |  |  |  |  |
| 4. | Decellularization |  |  |  |  |  |  |
| | Subjective evaluation plant scaffold 1. Transparency | + | ++++ | +++ | ++ | +++ | +++ |
| | 2. Handling Strength | + | ++++ | + | + | ++ | ++ |
| | 3. Patency | + | ++++ | ++++ | + | + | + |

**Fig 1:** Comparative evaluation of various medicinal plant leaves by decellularization process

## Dechlorophyllazation

After thawing, the leaf samples were transferred into ascending grades of alcohol 70%, 90% and 100% for 1 day each. After dechlorophyllazation, the leaf samples were transferred into distilled water for 24 hours followed by soaking in normal saline for 24 hours.

**Decellularization**

Before going to Decellularization process, the leaf samples were marked into different groups as showing in Table 1. Here we took five different groups for our research: one control, two 0.1% Triton and two 0.1% EDTA. This process was conducted for 5 days.

**Table 1:** The different steps followed during the study

| S. No | Steps of Protocols | Control | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|---|---|
| 1. | Soaking in DW for 24 h | √ | √ | √ | √ | √ |
| 2. | Frozen at-80℃ and thawed twice | √ | √ | √ | √ | √ |
| 3. | Soaking in 70 % ethanol for 24 h | √ | √ | √ | √ | √ |
| 4. | Soaking in 90 % ethanol for 24 h | √ | √ | √ | √ | √ |
| 5. | Soaking in 100 % ethanol for 24 h | √ | √ | √ | √ | √ |
| 6. | Soaking in DW for 24 h | √ | √ | √ | √ | √ |
| 7. | Soaking in NS for 24 h | √ | √ | √ | √ | √ |
| 8. | Decellularization | DW | 0.1% EDTA | 0.1% EDTA | 0.1% Triton | 0.1% Triton |
| 9. | Duration | 5 days | 5 days | 5 days | 5 days | 5 days |
| 10. | Rinsing Solution | DW | DW | DW | DW | DW |
| 11. | Duration | 24 hours | 24 hours | 24 hours | 24 hours | 24 hours |

DW-Distilled Water, NS-Normal Saline

**Vascular patency test**

As the handling strength was better in *Sauropus androgynus*. We have selected these plant leaves for further characterization. The decellulirized leaf samples of *Sauropus androgynus and Basella alba* were subjected for vascular patency test. Safranin (0.1%) was injected through the cannula attached to the petiole of each decellulirized leaves as described previously by Gershlak *et al.*, 2013.

**Histological technique**

The native decellularized leaves of *Sauropus androgynus* were further subjected to histomorphological studies. They were processed for routine histological technique and embedded in paraffin wax (Luna, 1968) [9]. Then, the samples were sectioned using semi-automatic microtome (Leica® RM2245, Leica Biosystems Pvt. Ltd., Singapore) to make 5-7µm thickness sections, the sections were carried on glass slides which were smeared with a swab of Mayer's egg albumin and then placed on the hot plate for 24 hours for the purpose of drying. The slides were stained using routine Haemotoxylen & Eosin (H & E) staining technique (Singh and Sulochana, 1996) [15]. The photographs were taken with a Nikon digital camera (Nikon®, MH 611 COOLPIX P5100, Japan) attached to CH 20i Olympus trinocular microscope. Samples of various medicinal plants leaves were cut into 1 cm thickness from the main vasculature of the leaves before cellularization and after decellularization for the tissue processing. Paraffin blocks were sectioned at 14 µm thickness and were stained using H&E (Luna, 1968) [9].

**Results and Discussion**

The natural structure of higher plants allows for the transport of nutrients via xylem and phloem to distal cells, e.g. from roots to leaves and leaves to roots or other leaves. To explore the potential for a plant based tissue engineered scaffold, perfusion decellularization (Ott *et al.*, 2008) [14]. Piper betel, Sauropus androgynus, Basella alba, *Azadirachta indica*, *Centella* asiatica, *Mentha spicata* all were kept in the deep freezer for 24 hours. After 24 hours leaves were taken back twice for thawing. After thawing observation was made in all leaves. The strength of the leaves was good in all the leaves except in betel and Basella alba. where the strength was reduced. Changes in colour were observed in all the leaves. After observation, all the leaves were kept in ascending grades of alcohol for dechlorophyllazation process for 24 hours. After dechlorophyllazation process, most of the chlorophyll was removed except in the betel (Fig 1). It was also observed for that there was better handling strength after decellularization process, especially with leaves of *Sauropus androgynus* when compared to others. The leaves Sauropus *androgynus, Basella alba and Centella asiatica* were found to be more transparent (Fig 1).

**Assessment of leaf vasculature patency post-decellularization**

A major advantage of using the decellularized leaf as a scaffold for tissue engineering is the innate vasculature. We thus wanted to assess whether the leaf vasculature remained intact and patent after the decellularization process. The saffranin stain (0.1%) was perfused through the cannula of decellularized *Sauropus androgynus* leaves. The stain perfused throughout the entirety of the leaf vasculature, with some minor leakages which were observed (Fig. 2). The perfusate also flowed into and through the smaller branches of the leaf vein (Movie1) indicating that the microvasculature of the leaf remained fairly intact.

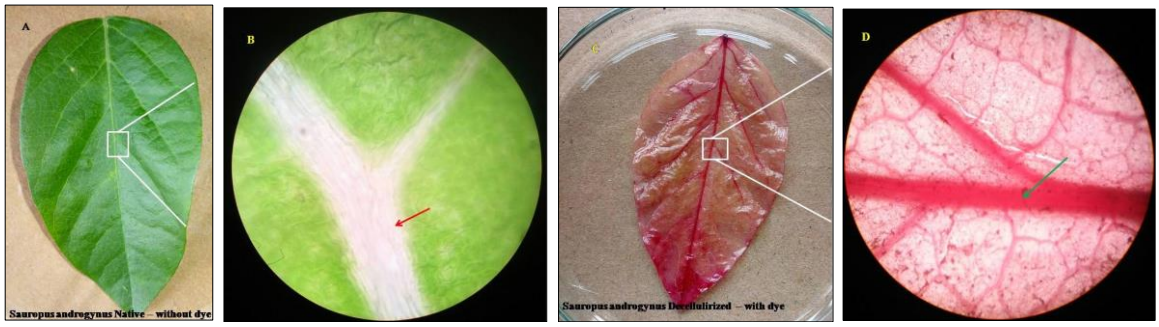



**Fig 2:** Vascular patency test before and after decellularization. Gross morphology of native leaf (A) and decellularized & dye injected leaf (C); (B) Native Leaf showing vascular structure (Red arrow); (D) decellularized leaf perfused with safranin stain which showed vascular patency (Green arrow)

**Histological evaluation**

In the present study, the efficiency of the decellularization process was evaluated by routine histological staining technique using native and decellularized leaves of *Sauropus androgynus*. The histological analysis of the decellularized leaf samples revealed that the current protocol removed the majority of cells of the native leaf as well as preserved the vascular structure. The native leaf was showing nuclei, where as nuclei were absent in decellularized leaf (Fig. 3). These observations correlated with the findings of the Gershlak *et al*, (2017) [7] for spinach leaves using safranin and fast green staining method.

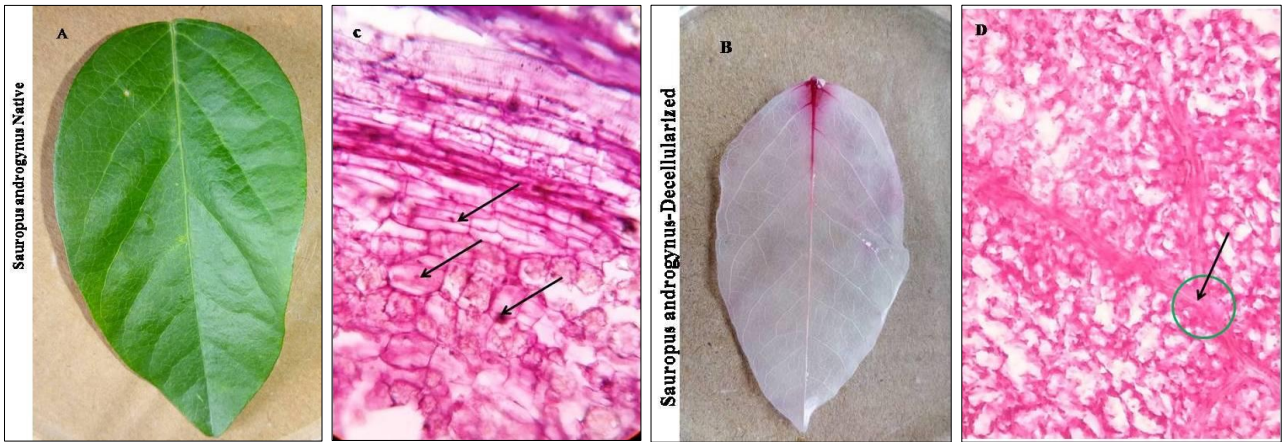



**Fig 3:** Anatomical characterization of plant scaffolds (*Sauropus androgynus*) before and after decellularization. Gross morphology of native leaf (A) decellularized leaf (B); (C) native and (D) dellularized leaf stained with H&E stain (400X): arrows indicating presence nuclei in native leaf and circle with arrow indicating vascular structure and absence of nuclei.

**Conclusion and future scope**

The morphological studies of medicinal plant leaves for their functional vascularity with aim to develop cellulose based prevascularized scaffolds is a novel approach for 3D cell culture and tissue engineering applications. Histological staining showed the loss of nucleic of materials from the plant tissue while maintaining both the structure and the differing native composition of the tissue, as noted when leaf was stained before and after decellularization. The decellularized plant leaf scaffolds will have potential clinical applications. The future scope of the study includes addressing the lack of outflow in plant vasculature and further determining plant tissue suitability for different tissue engineering applications.

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