



(RESEARCH ARTICLE)



Extraction of cellulose nano-crystals from *Polyalthia longifolia* and *Terminalia catappa* leaf litter and the synthesis of low-cost CNC-based hydrogel for articular cartilage

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GSC Biological and Pharmaceutical Sciences, 2021, 16(01), 019–026

Publication history: Received on 06 June 2021; revised on 10 July 2021; accepted on 13 July 2021

Article DOI: <https://doi.org/10.30574/gscbps.2021.16.1.0197>

Abstract

Due to the rise in demand for biodegradable and renewable materials, the synthesis of CNCs from lignocellulosic biomass opens up a new avenue for the creation and application of novel materials in nanotechnology. The CNC-based hydrogels appear to be a favorable material in various applications due to their excellent mechanical strength, biodegradability, biocompatibility, and low toxicity.

This work aimed to utilize the fallen leaves for the extraction of Cellulose Nano-crystals (CNC) from *Polyalthia longifolia* and *Terminalia catappa* leaf litter. Leaves mainly consist of cellulose hence used for the extraction of nanocellulose. Alkali treatment was performed with aqueous sodium hydroxide, followed by bleaching with aqueous sodium chlorite. Sulphuric acid hydrolysis was used for the extraction of CNC. The morphology, structure, functional groups, and crystallinity of the retrieved CNC were studied using a Transmission Electron Microscope (TEM), Fourier Transformed Infrared spectroscopy (FTIR), and X-Ray Diffraction (XRD).

The shape was rod-like for both *P. longifolia* and *T. catappa* and the CNC's crystallinity index was enhanced to 72.40% and 73.95%, respectively. The TEM micrographs revealed that the impurities present on the leaf fibres were successfully removed by alkali treatment and subsequent bleaching further purified the fibres, leaving behind mostly cellulose while the hemicellulose and lignin were removed, which was revealed in FTIR spectra.

The obtained CNC was used in the preparation of hydrogel by cross-linking with natural polymers like sodium alginate and gelatin. A Freeze-thawing process was carried out for the fabrication of hydrogel. The resulting hydrogel can be used as a substitute for cartilage applications.

Keywords: Cellulose Nano-crystal (CNC); Sulphuric Acid Hydrolysis; Transmission Electron Microscope (TEM); Fourier Transformed Infrared spectroscopy (FTIR); X-Ray Diffraction (XRD); Articular cartilage

1. Introduction

Over recent decades, fossil fuels have been increasingly scarce, green renewable resources arose wide attention, especially cellulose; the most abundant natural polymer [1]. Both rural and urban areas are burdened by the wastes that are generated from plants. Though the degradation process of the fallen leaves happens naturally but, it is a time consuming phenomenon. In urban settings the degradation process is difficult because the majority of soil is capped with concrete and results in the clogging of the drainage system. The disposal of this precious resource for making compost is generally done through burning which leads to air pollution or by dumping the litters into landfills [2, 3].

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Cellulose is mainly present in the cell wall of the plants and is composed of a linear chain of β -1,4-linked anhydro-D-glucose units [4]. Cellulose is composed of the both amorphous and crystalline regions and the CNCs are obtained from the crystalline region [5]. The extraction of the CNCs is done by hydrolysis process which results in the needle-like crystallites commonly called as Cellulose Nano-Crystals or Cellulose Nano-whiskers [6]. The CNCs are several nanometres in length and 10 – 30 nm in width having excellent biological properties such as biocompatibility, biodegradability, and non-toxicity [7]. Nano-crystals of cellulose also has important characteristics such as a specific surface, high aspect ratio (length/width), high crystallinity, and excellent mechanical properties which makes them useful in various applications like packaging, food additives, cosmetics, drug delivery systems, nanocomposite materials as reinforcing filler for the automotive industry, constructions, electronics and biomedical purposes [7, 8].

The CNCs have been extracted by various methods such as acid hydrolysis, alkali hydrolysis, enzymatic hydrolysis, TEMPO oxidation, steam explosion, ultrasonication, and grinding, high-pressure homogenization. Depending on the method of extraction in different compounds the physicochemical characteristics of the CNCs may differ [9, 10, 11, 12, 13].

The current investigation focuses on the extraction of CNCs from *Polyalthia longifolia* and *Terminalia catappa* leaf litters and used to prepare hydrogel. Since cellulose is rich with hydroxyl groups, it is used to perform physical cross-linking of hydrogen bonding to obtain gel formation. These gels are created by cross-linking hydrophilic polymer chains, which can absorb and retain large amounts of water in an aqueous environment without disintegrating. The bio-based hydrogels are biocompatible, non-toxic, and have a highly hydrated 3D porous structure, making them suitable for wound dressing, cell therapy, and tissue engineering. The fabrication of hydrogel networks is done via various processes which include homogenization (straight forward mixing), freeze-thawing process, UV/ion mediated cross-linking, free radical polymerization and 3D printing of natural (gelatin, alginate, chitosan) and synthetic (PVA, PAM, PEG) polymers [14, 15].

The CNC-based hydrogels can be used in medical implants due to its high mechanical strength. To date, CNCs have not been extracted from *Polyalthia longifolia* and *Terminalia catappa* leaf litters and therefore this study was undertaken. The samples were powdered using a grinder, then the powder was treated with sodium hydroxide for alkaline treatment to remove the impurities like pectin, wax and also helps in altering the hemicellulose and lignin structure. Sodium chlorite was used as a bleaching agent and sulphuric acid for hydrolysis. The characterization of the samples was performed via Transmission Electron Microscopy, Fourier Transform infrared spectroscopy, and X-Ray diffraction. The low-cost hydrogel is prepared using the extracted CNCs in combination with other natural polymers (sodium alginate and gelatin). Freeze-thawing technique was followed and calcium chloride was used as a cross-linking agent. The CNC-based hydrogels improves the mechanical properties of hydrogels, which makes them a promising material to use for cartilage applications, especially for the easy and rapid repair of injured cartilage.

2. Material and methods

2.1. Materials

The biomasses used in this study are *Polyalthia longifolia* and *Terminalia catappa* leaf litters collected from the soil surface during the summer season in R. V. College of Engineering campus.

Chemicals: Sodium hydroxide (NaOH), Sodium Chlorite (NaClO_2), Sulphuric acid (H_2SO_4), Sodium alginate, Gelatin, Glycerol, Sodium Chloride (NaCl), Calcium Chloride (CaCl_2), 12kDa dialysis membrane were provided by R. V. College.

2.2. Sample processing

First, the leaf litters of *P. longifolia* and *T. catappa* were collected separately and washed with tap water to remove the dirt. The leaves are then rinsed with distilled water and air dried for 24hrs. Milling or grinding are common methods for reducing particle size to facilitate material handling and increase the surface-to-volume ratio. So, the dried samples were crushed by hand and dried in the oven at 90°C. After drying, the samples were ground using a mixer grinder and sieved through a #60 mesh sieve. The samples were stored in an airtight container separately and stored at room temperature.

2.3. Extraction of Cellulose fibers

The cellulose fibres were extracted from the samples using an alkali treatment, which consisted of dissolving 50g of *P. longifolia* leaves powder in 1 litre of 1M (4% w/v) NaOH. The mixture was kept under stirring conditions at 80°C for 2 hours. The alkali treatment was repeated 3 times and washed several times with distilled water to remove the alkali

contents. Also, the pH was neutralized. The residues were then oven-dried at 50°C for 24 hours. The same procedure was carried out for the *T. catappa* sample as well. Then, bleaching treatment was carried using 2% w/v NaClO₂ at 100°C for 1 hours under stirring conditions. The bleaching treatment was repeated several times till the colour changed to white. In the end, the fibres were washed with distilled water to neutralize the pH and oven-dried at 50°C for 24 hours. The same procedure was carried out for *T. catappa* sample as well.

2.4. Acid hydrolysis

The extracted cellulose fibres were hydrolysed to obtain Cellulose nano-crystal through the acid hydrolysis method. 10g of the extracted cellulose fibres were added into 200 ml of 64% w/w sulphuric acid and was hydrolysed at 45°C for 1 hour at stirring condition. The reaction was stopped by adding 5 fold distilled water and then being centrifuged at 10,000 rpm for 10 mins and the gel suspension was collected. Filtering the collected gel suspension via a dialysis membrane soaked in distilled water neutralised it. The dialysis membrane used was of 12kDa molecular weight cut off. Finally, the neutralized CNC suspensions were then sonicated by using an ultrasonic sonicator for 20 mins and then stored in a refrigerator at 4°C.

2.5. CNC-based Hydrogel

In a beaker containing distilled water, 1% sodium alginate was dissolved at 60°C for 2 hours kept on a magnetic hotplate stirrer. In another beaker, 1% of homogeneous solution of gelatin was prepared at 35°C under constant stirring. The solution was then heated to 60°C stirring continuously. To this, 30% glycerol and 0.2% NaCl were added. This mixture was then poured into the above beaker containing the Sodium alginate solution.

Finally, 2% of CNC was heated at 40°C by stirring continuously and added to the prepared mixture under stirring for 2 hours until a homogenous dispersion was obtained. After that, the mixture was cast and dried in a 40°C oven for two days. The dried mixture was then immersed for 1 hour in a 1% CaCl₂ aqueous solution to allow cross-linking. To eliminate the unbound cross-linking agents, the samples were rinsed with distilled water. Finally, froze at -20°C for 24 hours and then thawed at room temperature. The Freeze-thawing process was repeated 4 times to obtain cross-linked sponges.

2.6. Characterization

The shape and size of the CNCs are examined using Transmission electron microscopy (TEM). The functional groups in the samples were investigated using Fourier Transform Infrared spectroscopy and the XRD patterns of the samples were recorded to calculate the crystallinity index (CrI) by using the equation: $CI (\%) = (I_{crys} + I_{amp}) / I_{amp} * 100$ where, $I_{crys} + I_{amp}$ is parts of crystalline and amorphous at $2\theta = 23^\circ$ and I_{amp} is the intensity peak of the amorphous region at $2\theta = 16^\circ$ [16, 17, 18].

3. Results and discussion

3.1. Sample Collection and Processing



Figure 1 Collected Leaf Samples (a) *Polyalthia longifolia* and (b) *Terminalia catappa*

Figure 1 Shows the images of the raw materials used for the study. The leaf samples were collected, washed and kept for drying.



Figure 2 Samples Powdered and Sieved

Figure 2 Shows images of powdered and sieved samples of (a) *Polyalthia longifolia* and (b) *Terminalia catappa*

3.2. Extraction of Cellulose fibres

3.2.1. Alkaline Treatment



Figure 3 Alkali Treated

Figure 3 Shows the dried (a) *Polyalthia longifolia* and (b) *Terminalia catappa* samples after alkali treatment

3.2.2. Bleaching Treatment



Figure 4 Bleached Samples

Figure 4 Shows the dried cellulose fibres of (a) *Polyalthia longifolia* and (b) *Terminalia catappa* obtained after bleaching treatment

3.3. Acid Hydrolysis

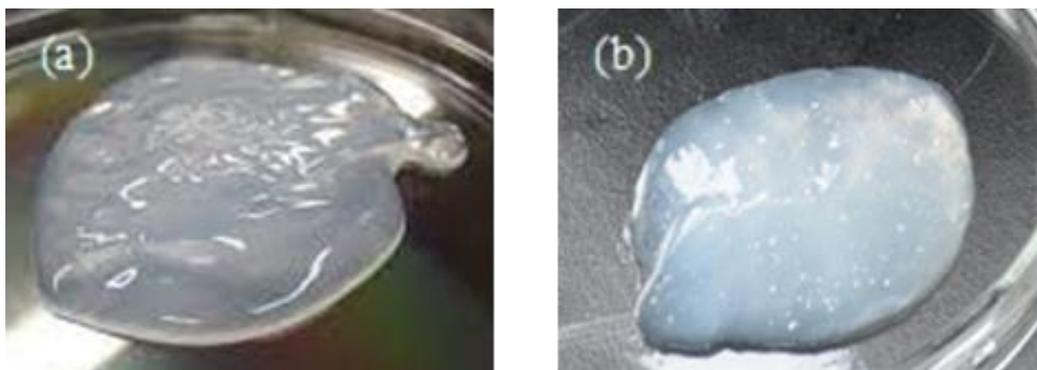


Figure 5 Cellulose Nano-cystals

Figure 5 Shows the Cellulose Nano-crystals obtained from (a) *Polyalthia longifolia* and (b) *Terminalia catappa* leaves

3.4. CNC Based Hydrogel



Figure 6 CNC based Hydrogel

Figure 6 Shows the insoluble gel of CNC-based hydrogel

3.5. Characterization

3.5.1. Transmission Electron Microscope

The characteristic size and shape of cellulose nano-crystals hydrolysed by acid hydrolysis is shown in Figure 7 for (a) *Polyalthia longifolia* and (b) *Terminalia catappa*. The CNCs were discovered to be rod-like in shape, with an average ($length \times width$) of $(296.02 \pm 36.45 \text{ nm} \times 5.79 \pm 2.08 \text{ nm})$ and $(258.85 \pm 32.64 \text{ nm} \times 4.81 \pm 4.47 \text{ nm})$ for *P. longifolia* and *T. catappa*, respectively. It was observed that there were no impurities present in both samples. The Aspect ratios are mentioned in the below Table 1.

Table 1 TEM characterizations of the average dimensions

Samples	Length (nm)	Width (nm)	Aspect Ratio (l/d)
<i>P. longifolia</i>	296.02 ± 36.45	5.79 ± 2.08	51.12
<i>T. catappa</i>	258.85 ± 32.64	4.81 ± 4.47	53.81

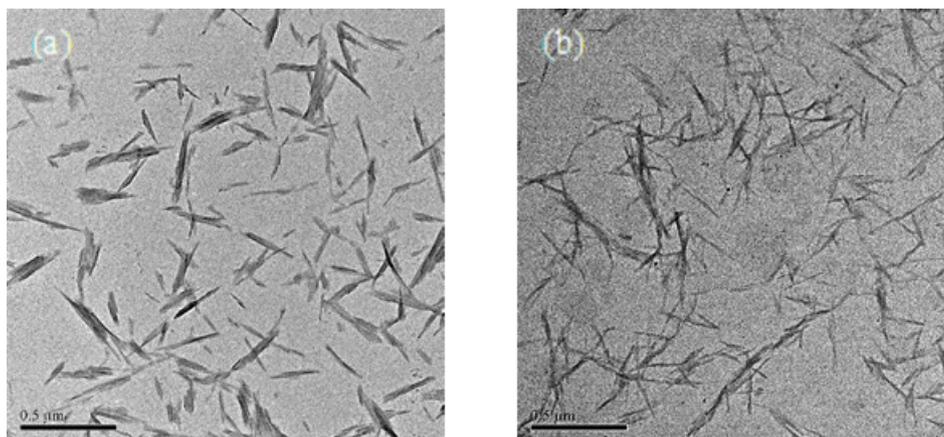


Figure 7 TEM Images

3.5.2. Fourier Transmission Infrared Spectroscopy

Figure 8 shows the FTIR spectra images. It was found that cellulose existed in all of the samples. The pure cellulose with characteristic peaks of $-OH$, $C-H$, and $C-O$ stretching vibrations were observed at 3325cm^{-1} , 2854cm^{-1} , 1643cm^{-1} and 3390cm^{-1} , 2890cm^{-1} , 1630cm^{-1} for (a) *P. longifolia* and (b) *T. catappa* respectively. The glycosidic deformation was seen at 900cm^{-1} and 882cm^{-1} for *P. longifolia* and *T. catappa* respectively, as shown in Table 2.

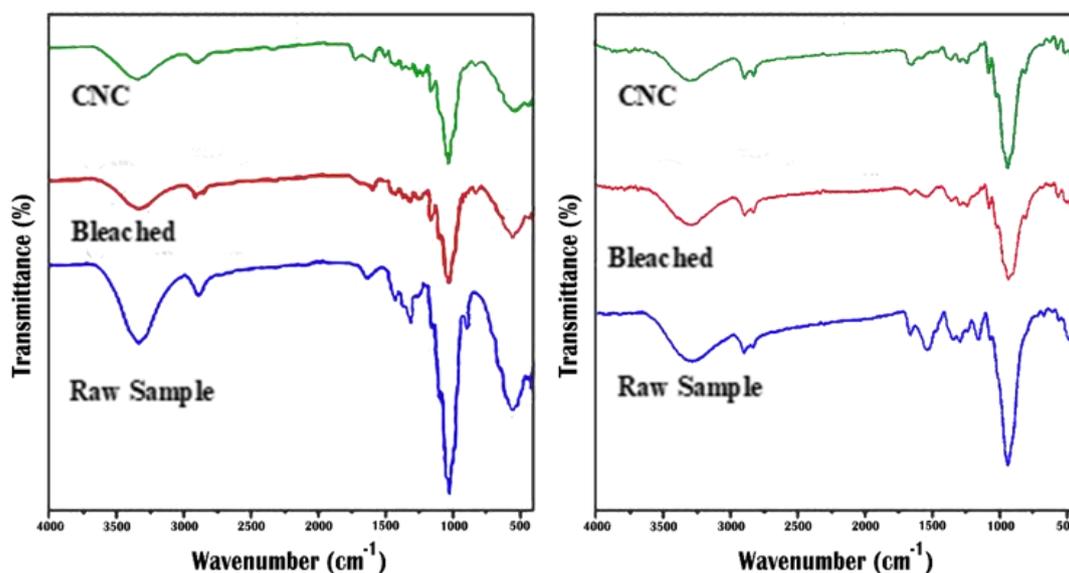


Figure 8 FTIR Spectra

Table 2 FTIR characterizations of the functional groups

Assignments	Reported Cellulose (cm^{-1})	
	<i>P. longifolia</i>	<i>T. catappa</i>
$-OH$ groups stretching vibrations	3325	3390
$C-H$ stretching vibrations	2854	2890
H_2O absorbed	1643	1630
CH_2 bending vibrations	1436	1450
$C-O-C$ glycosidic band stretching vibration	1160	1154
$C-H$ rock vibration	900	882

3.5.3. X-ray Diffraction

The diffraction patterns were seen in the XRD spectrum of *P. longifolia* and *T. catappa* bleached and hydrolysed CNC. Figure 9 showing X-ray diffraction patterns after being bleached and hydrolyzed for (a) *Polyalthia longifolia* samples shows three peaks at $2\theta = 16.2^\circ$, 23.0° , and 34.8° . The crystallinity index of the hydrolyzed CNC was increased to 72.40%. In contrast, (b) *Terminalia catappa* samples show three peaks at $2\theta = 16.0^\circ$, 23.2° , and 34.9° and the crystallinity index of the hydrolyzed CNC was increased to 73.95%.

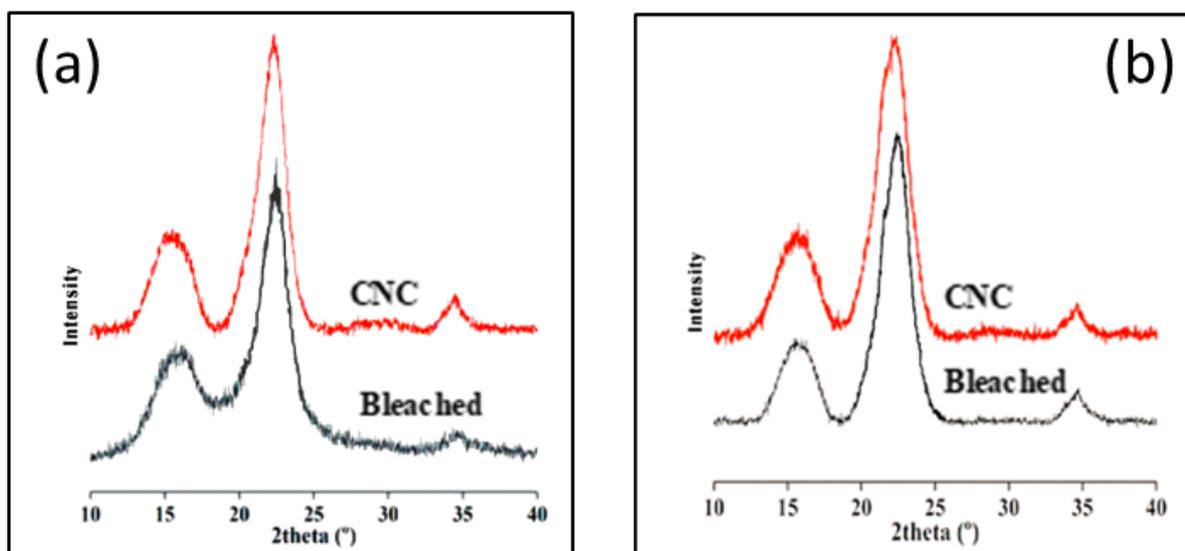


Figure 9 X-Ray Diffraction Patterns

4. Conclusion

In this study, Cellulose nano-crystals were successfully extracted from *Polyalthia longifolia* and *Terminalia catappa* leaf litter via sulphuric acid hydrolysis. The Non-Cellulosic components were extensively removed by alkali treatment and bleaching treatment. The morphological study confirms that the chemical treatment used is appropriate to obtain CNC with a high aspect ratio of 51.12 and 53.81 also the CNCs exhibited rod-like shapes with crystallinity indexes of 72.40% and 73.95% for *P. longifolia* and *T. catappa* respectively. After the chemical treatment, the FTIR spectra showcased the elimination of hemicellulose and lignin. As a result, the *Polyalthia longifolia* and *Terminalia catappa* leaf litter can be used as a cost-effective source for the production of CNC which can be used as a reinforcing agent in various applications.

The extracted CNC from *Polyalthia longifolia* and *Terminalia catappa* leaf litter was successfully used in the preparation of hydrogel. CNC, in addition to sodium alginate, gelatin and glycerol were mixed together and for cross-linking, CaCl_2 was used. The 3D hydrogel was fabricated by a freeze-thawing process. The prepared hydrogel is biocompatible, elastic, viscoelastic and flexible in nature. For people suffering from osteoarthritis, it can be used as articular cartilage.

Compliance with ethical standards

Acknowledgments

I acknowledge my sincere gratitude to my guide, Dr. Pushpa Agrawal, Professor, Department of Biotechnology, R.V. College of Engineering, Bengaluru, India for her guidance and constant encouragement given throughout the course of the project. I would like to convey my gratitude to Department of Biotechnology, R.V. College of Engineering for providing me with the lab facility to carry out the experiments.

Disclosure of conflict of interest

Both the authors, Drathi U K and Pushpa Agrawal declares that no conflict of interest in this article.

Statement of ethical approval

The present research work does not contain any studies performed on animals/humans subjects by any of the authors.

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