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Amrit Research Journal December 2024, Vol. 5, Issue 1, 40-48
ISSN: 2738-9553
RESEARCH ARTICLE

Incorporation of Silver Nanoparticle in Cellulose Nanocrystal Extracted from *Eulaliopsis binata* Leaf to Enhance Its Antimicrobial Activity

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(Received: August 7, 2024; Received in Revised form: October 6, 2024; Accepted: October 22, 2024; Available online)

DOI: https://doi.org/10.3126/arj.v5i1.73523

Highlights

- Cellulose nanocrystal (CNC) was prepared from the leaves of *Eulaliopsis binata*
- Cellulose was extracted via alkalization, acetylation, and acid-hydrolysis
- AgNPs were prepared using the aqueous extract of *Cannabis sativa* and silver nitrate
- AgNPs were incorporated into CNC to produce CNC@AgNPs
- Both NMs showed antimicrobial activity against *E. coli*, *B. subtilis*, & *C. albican*

Abstract

Cellulose, one of the most abundant natural biopolymers, is widely regarded as a sustainable material and is primarily found in the cell walls of plants. This study focuses on the isolation of cellulose nanocrystals from the leaves of Eulaliopsis binata. The extraction of cellulose was performed using established chemical methods, including pre-alkalization, alkalization, acetylation, and acid hydrolysis. Silver nanoparticles were prepared through a green synthesis method and subsequently impregnated into the extracted cellulose. The resulting nanoparticle-impregnated cellulose was analyzed using Fourier-transform infrared spectroscopy (FTIR) and X-ray diffraction (XRD) techniques. An antimicrobial assessment of the silver nanoparticle incorporation into cellulose nanocrystals revealed their effectiveness against selected microorganisms.

Keywords: *Eulaliopsis binata, cellulose, alkalization, acetylation, nanocrystal, antimicrobial*

Introduction

Cellulose is a natural polysaccharide consisting of repeating β-D-glucose monomer units linked by β-(1,4)-glycosidic bonds, forming a linear polymer with the chemical formula, $(C_6H_{10}O_5)_n$, where *n* represents the number of glucose units in the polymer chain. It is abundantly found in plants, algae and certain bacteria [1]. Cellulose is a primary structural component of plant cell walls and is also produced by some bacteria and algae. Cellulose molecules are linear, unbranched chains with strong hydrogen bonding which impart then high mechanical strength and resistance to chemical degradation [2]. Biomass-derived cellulose enables multiple functions and transformative applications due to its specific structure and versatile applications [3]. It has been studied and used for many years, and it will continue to be a vital raw resource for food, paper, and additives in the

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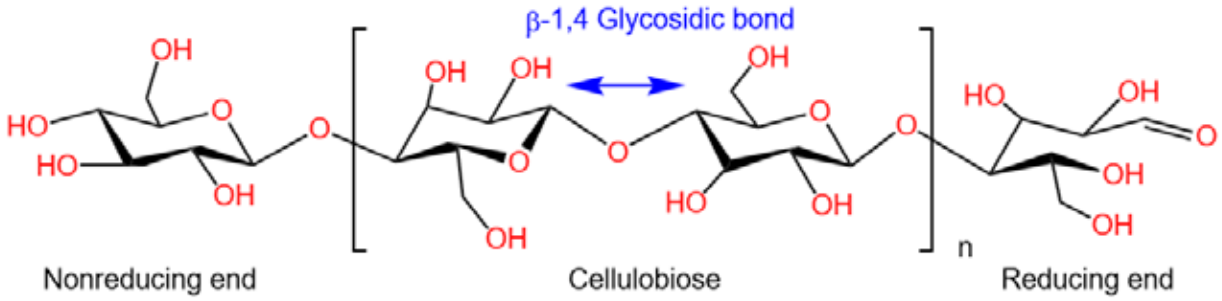
pharmaceutical and optical sectors. The main sources of cellulose are the most plentiful biomass resources such as wood, rice husk, maize husk, banana peel, barley husk, rice straw, etc. [4]. The molecular structure of cellulose indicating reducing and non-reducing ends is shown in scheme 1.



Scheme 1: Molecular structure of cellulose fiber showing β-1,4 glycosidic linkage, reducing and non-reducing ends [5].

Cellulose polymers are being used in the preparation of note currency, napkins, tissue paper, writing paper, wrapping paper, paper towels, and paper bags. These paper-made materials are being used every day. Long-term storage and uses of these materials could get attacked by various fungi and bacteria which could be a serious issue for human health. For health-beneficial issues, it is better to have antimicrobial properties on those types of commercial products. The antimicrobial activities of such products can be enhanced either by selecting suitable raw materials with inherent antimicrobial properties or by incorporating nanoparticles possessing these properties [6]. The former one is limited because of the abundance of resources while the latter is the more convenient and suitable one. Researchers have claimed that AuNPs, AgNPs, CuNPs, CuONPs, Ag_2ONPs, Cu-ZnNPs, Ag-CuNPs, etc. have good antimicrobial activities against both Gram-positive and Gram-negative bacteria as well as some fungi [7-11]. However, AgNPs are more effective against a wide variety of microbes as well as convenient for impregnation in the nanofibers [3, 12]. These nanoparticles release silver ions when enter the body of host pathogens and then control their growth either by rupturing their cell membrane or by controlling their DNA replication [13]. Green synthesis of AgNPs is more common and in this experiment, *Cannabis sativa* extract was used as a reducing and capping agent. Thus, synthesized nanoparticles were incorporated into the cellulose nanocrystals obtained from *Eulaliopsis binata*. It is a native Nepali grass called Babio (Figure 1). It is a perennial grass with long leaf blades 30-80 cm and a flowering stem 60-90 cm belonging to the family Poaceae. This plant was chosen as the source of the cellulose nanocrystal for this investigation. Nevertheless, since very ancient times, it has been utilized as a source of cellulose for paper mills; yet the enhancement of its quality in terms of microbial action has not been accomplished.

Fig 1: *Eulaliopsis binata* plant leaves

Materials and Methods

Chemicals and Instruments

All the reagents and chemicals used during the experiment were of reagent grade and used directly without further purification. Chemicals like Ethanol (99.9%, Changshu Chemicals), Benzene (98%, Thermo Scientific), sodium hydroxide (NaOH, 97%, Fizmerk Chemicals), Acetic acid (99.5%, Qualikems), Nitric acid (71%, sp.gr. 1.41, Fine Chemicals) Distilled water, silver nitrate and Sulphuric acid (98.0%, sp.gr. 1.84, Merck) were used in the research work. Glass wares like funnels, beakers, weighing machines (KERN and Sohn GmbH), pH meter, and herbal disintegrator (Model FW 177), FTIR (Perkin Elmer 10.6.2) and XRD (Rigaku diffractometer, Cu Kα, $\lambda = 1.5406$ Å) were used to complete this project.

Extraction of Cellulose Nanocrystals

Eulaliopsis binata leaves were collected from Ghorahi Sub Metropolitan City, Nepal (Latitude: 28.0403 N, Longitude: 82.4860 E). Collected leaves *were* shade dried and the dried sample was ground into fine powder form by grinding mill. The sample powder was then stored in the moisture-free desiccator. 10 g of powdered sample was weighed in a watch glass and then transferred into a beaker. Then 50 mL ethanol and 100 mL toluene were mixed into this sample and left for 24 hours. The sample was filtered, and residue was taken in a round bottom flask and mixed with 2% NaOH solution, and left for 12 hours at 40 °C. The mixture was filtered and then the residue was mixed with 7.5% NaOH solution. The sample was refluxed for 90 minutes to remove hemicellulose and lignin. The refluxed sample was cooled for some time and then filtered. Then residue was taken in a beaker and washed until pH was maintained at 6. Then the content was filtered. The residue was taken and transferred to a round bottom flask and mixed in 60 mL glacial acetic acid and 10 mL concentrated nitric acid. Again, the sample was refluxed for 30 minutes. The reflux solution was cooled and filtered. Then the residue was treated with sulphuric acid at 45 °C for 40 minutes under continuous stirring. The mixture was filtered, and the residue was taken. This residue was washed with distilled water repeatedly till the solution became turbid (white gelatinous cellulose nanocrystal Tyndall in the solution). The sample was centrifuged and filtered. The residue was taken and dried. The sample was collected and weighed out. This product was used for characterization. The final product was cellulose nanocrystals (CNCs).

Green Synthesis of Silver Nanoparticles

Silver nanoparticles were synthesized using an aqueous extract of *Cannabis sativa* and a precursor solution of silver nitrate solution. For the synthesis of nanoparticles, 30 mL of plant extract was taken in a beaker in which 10 mL of 0.1 N $AgNO_3$ and 30 mL of distilled water were added. The mixture was kept constantly stirred on a magnetic stirrer for 4 hours. The UV spectral measurement was carried out in each 30 minutes time interval to monitor the formation of nanoparticles. Visual observation reflects the color changing of the solution from grey to dark. The dark-colored solution was then centrifuged for 5 min at 4000 rpm. Silver nanoparticles of solutions settled down in the bottom of centrifuge tubes and those were collected in the watch glass. Collected nanoparticles were washed with ethanol and dried. Finally, silver nanoparticles were collected and stored in a vile. Then the silver nanoparticles were characterized.

Incorporation of Silver Nanoparticles

Silver nanoparticles were incorporated in the cellulose nanocrystal by dispersion method. For this, 0.05 g of silver nanoparticles and 2 g of cellulose were dispersed in 50 mL water with continuous stirring on a magnetic stirrer for 30 minutes. The sample was filtered with Whatman filter paper & residue was taken on the watch-glass and kept in a safe & clean place for 2-3 days. The dry cellulose nanocrystal incorporated silver nanoparticles (CNC@AgNPs) were obtained. Then the materials were characterized.

Physico-chemical characterization

The dry cellulose nanocrystals obtained were quantitatively determined and then the functional groups associated with them were determined using FTIR measurement. The crystallinity of the cellulose was also determined by XRD measurement. After the successful impregnation of AgNPs, it was further characterized by XRD. Similarly, the antimicrobial activity of nanoparticle-impregnated cellulose nanocrystals was tested for selected microbes by disc diffusion method.

Results and Discussion

Yield

The collected cellulose nanocrystal was dried in a hot air oven below 100 ºC. The weight of the dried nanocrystal was measured using a 3-digit electronic balance and the obtained weight of dry mass is tabulated in Table 1. The yield % of the cellulose nanocrystal was also calculated. The average weight of cellulose nanocrystal formed from the plant fiber is 31.34%. Based on the results, the plant material may be classified as a cellulose-rich raw material with prospective use in the papermaking industry.

Table 1: Cellulose nanocrystal of the *Eulaliopsis binata* Plant

Measurement samples	Sample taken (g)	Obtained nanocellulose (g)	% of nanocellulose	Average % of nanocellulose
Sample 1	10	3.085	30.85	31.34
Sample 2	10	3.182	31.82	

Visual Characterization

At the end of the extraction procedure, the white gelatinous cellulose dispersed in a dilute acid medium was obtained. The gelatinous cellulose fiber dispersed in an aqueous medium is shown in Figure 2(a). After filtration, washing with distilled water followed by drying, a solid mass of cellulose nanocrystal was obtained as shown in figure 2(b).

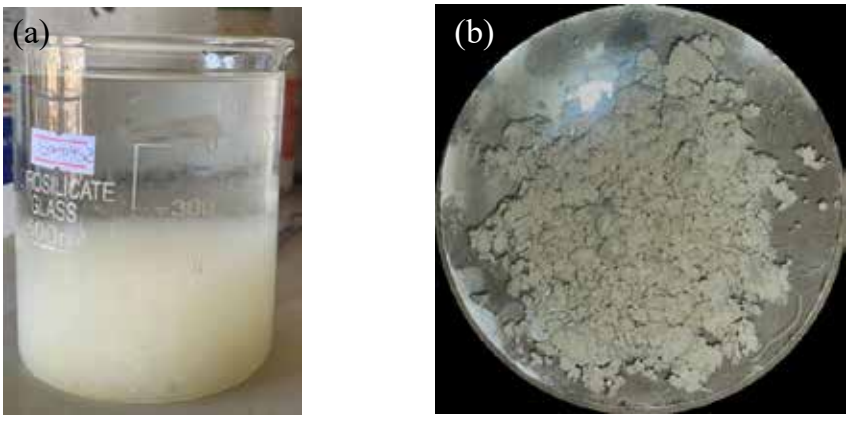


Fig 2: a) Cellulose nanocrystal in dispersed condition and b) cellulose nanocrystal in dry form.

Functional group analysis

The functional group analysis of the material was carried out by attenuated total reflectance (ATR) mode of Fourier transform infra-red (Perkin Elmer Spectrometer 10.6.2 version, FTIR) analysis. FTIR spectrometric determination of cellulose was carried out at the Department of Chemistry, Amrit Campus, Kathmandu, Nepal. The background correction was carried out using isopropanol. All the spectral data were collected from 450-4000 cm^{-1} cutoff range with 4 cm^{-1} resolutions.

The FTIR spectra of the cellulose nanocrystal extracted from the *Eulaliopsis binata* stem are shown in Figure 3(a). It is evident that the strong peak observed around 3300 cm^{-1} is the characteristic peak for the stretching vibration of O-H bond and the peak around 2890 cm^{-1} is the stretching vibration of C-H bonds in polysaccharides [14]. The strong peaks in the range of 1750-900 cm^{-1} are typical for cellulose identification. The peak located at 1635 cm^{-1} is due to unsaturated double-bond carbon. The strong peaks at 1164, 1159, 1034, and 897 cm^{-1} are due to stretching and bending vibrations of $–CH_2$, -C-H, -O-H, and C-O bonds in cellulose [14-16].

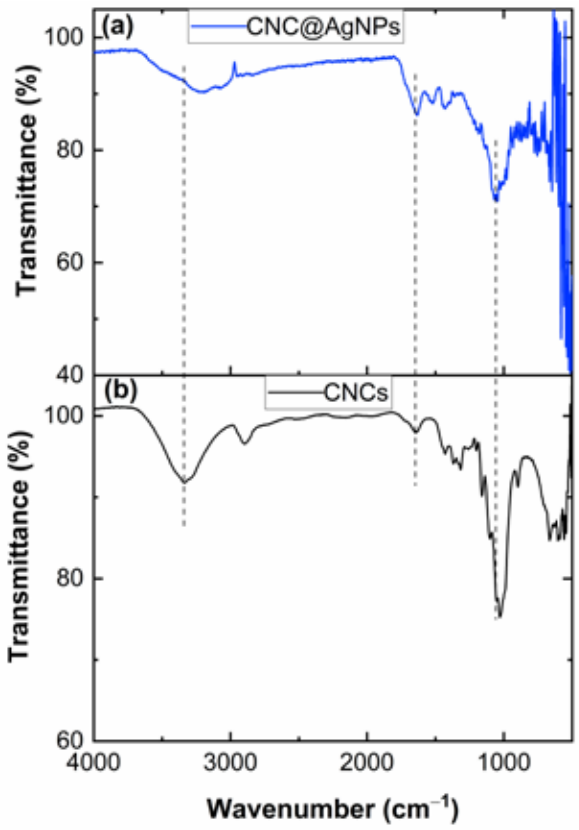


Fig 3: a) FTIR spectra of a) cellulose nanocrystals (CNCs) and b) nanoparticle-incorporated cellulose nanocrystals (CNC@AgNPs)

The FTIR spectra of nanoparticle-incorporated cellulose nanocrystals are shown in Figure 3(b). It is found that there is characteristic band of –O-H stretching vibration around 3200 cm^{-1}. Also, at 1636 cm^{-1} due to unsaturation i.e. due to the carbon-carbon double bond of polysaccharides, likewise at 1048 cm^{-1} is due to –O-H bending vibration. All the observed peaks in the range of 2850-3650 cm^{-1} are the characteristic peaks for stretching vibration of O-H and C-H bonds in polysaccharides [14]. The broad peak at 3391 cm^{-1} is characteristic of the stretching vibration of the O-H group on polysaccharides in pure cellulose nanocrystal which is shifted to 3200 cm^{-1} in nanoparticle-incorporated cellulose. This implies that the -OH group is occupied by nanoparticles in the composite system. The peaks in the region of 1750-900 cm^{-1} are typical for cellulose identification. The peak located at 1635 cm^{-1} is due to unsaturated double-bond carbon. The absorbance peaks around this area are also shifted to a lower energy area indicating the occupying nature of the functional groups with nanoparticles. The strong peaks at 1164, 1159, 1034, and 897 cm^{-1} are due to stretching and bending vibrations of $–CH_2$, -C-H, -O-H, and C-O bonds in cellulose [14, 16]. The characteristic peak of cellulose around 1035 cm^{-1} is not shifted, the rest of all are shifted in the nanoparticle incorporated cellulose. This shows that AgNPs are successfully incorporated in cellulose nanocrystals.

XRD characterization

Diffraction occurs when a collimated beam of electromagnetic waves with a wavelength comparable to the interatomic distances interconnected with the periodic array of molecules in a crystal. X-ray diffraction (XRD) is the most widely used method to determine the crystallinity of the materials [17]. X-ray diffraction (XRD) is a versatile non-destructive analytical technique used to analyze physical properties such as phase composition, crystal structure, orientation of powder, solid, and liquid samples. The XRD patterns of cellulose are illustrated in Figure 4. The diffraction pattern of all cellulose shows a highly intense peak around 23° which is the characteristic peak of cellulose crystal. Similarly, the supporting peaks around 16° and 34° resemble the cellulose crystals. The intense peaks at 16°, 23°, and 34° correspond to the crystal planes (110), (200), and (004), respectively [18,19].

The diffraction peaks observed at 27.7, 32.2, 38.1, 46.2, 54.7, 57.3, and 65.1 belongs to (210), (122), (111), (231), (200), (220), and (220), respectively, are the crystal planes of AgNPs. Peaks observed at 27.7, 54.7, and 57.3 have seemed to be silver oxides. However, some of the literatures have also reported the similar crystal pattern of AgNPs on those diffraction angles [20, 21]. The average grain size of AgNPs was determined using Debye Scherer's formula and found to be 12.32 nm.

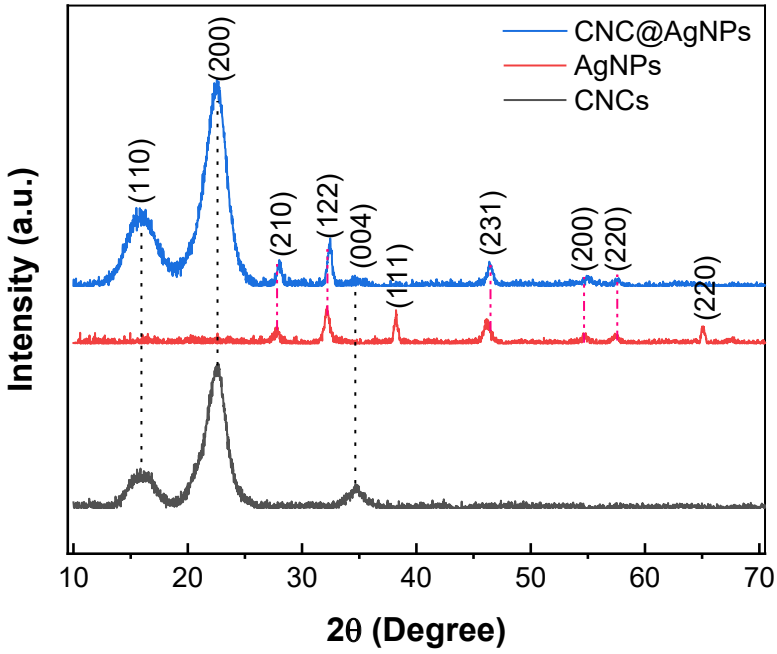


Fig 4: XRD pattern of Cellulose nanocrystals (CNCs), Silver nanoparticles (AgNPs) and Silver nanoparticle incorporated cellulose nanocrystals (CNC@AgNPs)

X-ray diffraction analysis was carried out to confirm the incorporation of crystalline silver nanoparticles on the cellulose nanocrystal. XRD pattern of nanoparticle-impregnated cellulose crystals is shown in Figure 4. The XRD pattern showed several Braggs reflections that may be indexed based on the face-centered cubic structure of silver nanoparticles as well as due to cellulose nanocrystals. The intense peaks at 16°, 23°, and 34° of cellulose nanocrystal as well as the intense peaks at 28°, 32.4°, and 46.8° corresponding to (210), (122) and (231), respectively for silver nanoparticles (JCPDS, file No. 04-0783) [22, 23]. All the Braggs reflections are based on the face-centered cubic structure of silver nanoparticles.

All the XRD patterns have numbers of Braggs diffractions confirming the existence of crystals in the sample. The XRD patterns of cellulose and silver nanoparticles resemble each other. The main common peaks at 2θ = 16°, 23°, and 34° corresponding to the (110), (200), and (004), respectively are due to cellulose nanocrystals. These resembled patterns show that there is no shifting of cellulose nanocrystalline peak position. This implies that impregnated AgNPs did not influence the crystal structure of cellulose. However, there are additional peaks in nanoparticles incorporated in cellulose suggesting silver nanoparticles get imparted in cellulose crystals.

Antimicrobial activity

The antimicrobial activity of silver nanoparticles and silver nanoparticle-incorporated cellulose nanocrystals has been studied and the zone of inhibition (ZOI) found is presented in Table 2. The antimicrobial activity of these materials was studied against two American Type Culture Collection (ATCC) bacteria and a fungus using the agar disc diffusion method. Table 2 shows the zone of inhibition of silver nanoparticles and silver nanoparticle incorporated cellulose nanocrystal when spread on Gram +ve and Gram -ve bacteria and fungus incubated for 12 h. The ZOI shown by AgNPs is little higher than CNC@AgNPs for Gram +ve bacteria and fungus but it is same for Gram -ve bacteria. The ZOI shown by positive control Kanamycin was 1.05 cm.

Table 2. Zone of inhibition shown by AgNPs and AgNPs incorporated cellulose nanocrystal

Microorganisms	Reference Code	Zone of Inhibition (cm)	
		AgNPs	CNC@AgNPs
Bacillus subtilis (Gram +ve)	ATCC 6051	0.76	0.70
Escherichia coli (Gram –ve)	ATCC 8739	0.80	0.80
Candida albicans (Fungus)	ATCC 2091	0.90	0.85

This study demonstrated the antimicrobial activity of silver nanoparticles as well as silver nanoparticle-incorporated cellulose nanocrystals (CNC@AgNPs) against Gram-positive, Gram-negative bacteria, and fungi by agar disc diffusion method. The result shows that silver nanoparticle exhibited a slightly higher zone of inhibition (ZOI) than CNC@AgNPs for *Bacillus subtilis* bacteria and *Candida albicans*. However, in the case of *Escherichia coli*, the ZOIs exhibited by both AgNPs and CNC@AgNPs are same. These findings show the potential of both AgNPs and CNC@AgNPs as effective antimicrobial agents with slight variations in performance depending on the type of microorganism.

Conclusions

The lignocellulose substance, cellulose, was effectively isolated from the leaves of the *Eulaliopsis binata*. Characteristic stretching and bending vibration in FTIR spectra shown by the functional group associated with cellulose confirmed the successful extraction of cellulose. The crystalline peaks in XRD revealed that the extracted cellulose is in crystalline nature. The distinct peaks of cellulose nanocrystals as well as AgNPs in XRD measurement confirmed the successful impregnation of nanoparticles in the cellulose nanocrystals. Nanoparticle-incorporated cellulose demonstrated strong antibacterial action against a variety of microorganisms i.e. *Escherichia coli* (Gram-negative bacteria), *Bacillus subtilis* (Gram-positive bacteria), & *Candida albicans* (Fungus). All in all, results show the potential of AgNPs and CNC@AgNPs as effective antimicrobial agents.

Acknowledgements

The authors are grateful to Mahendra Multiple Campus, TU, Dang Nepal, and Amrit Campus, TU, Kathmandu Nepal, for laboratory support, Himalayan Research Center for antimicrobial tests, JNCSR, India for XRD measurements.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

Data will be available upon legal request to the corresponding authors.

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