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Original Research Article

Modification of Nano Organic Material Surface Using Lemon as an Antibacterial Agent for Fish

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Abstract: The modification of nanocellulose surfaces using lemon extracts as an antibacterial agent for fish applications presents a promising avenue for enhancing fish health. This approach leverages the natural antibacterial properties of lemon, which can be integrated into nanocellulose, a biopolymer known for its biodegradability and mechanical strength. The following sections outline the key aspects of this modification process. Because cellulose's surface has a large number of reactive groups, or hydroxyl groups, it is easily functionalized with other functional groups, including amines, carboxylic acids, and aldehydes, to produce a variety of characteristics. Furthermore, the variety of substances that can be grafted onto cellulose's structure, including proteins, polymers, metal nanoparticles, and antibiotics, is increased by the material's ease of surface modification. Cellulose nano-/microfibrils and nanocrystals are utilized in numerous research projects as a vehicle for antibacterial drugs. this investigation on nanocellulose. Using a sustainable and ecofriendly analytical method, nanoparticles made from natural materials and physical technical processes were created without the use of chemicals during the production or extraction processes. its usage as a reinforcing agent; the production of nanocrystalline cellulose and its application in nanocomposites. The antimicrobial properties of Citrus limonum fruit juice extract were examined. After dissolving in a nonionic surfactant solution, an antibacterial drug that is not highly water soluble was added to cellulose nanocrystals. This study describes a novel attempt to use lemon, an environmentally safe bactericidal agent, to chemically cross-link antimicrobial nanocellulose. The findings of antimicrobial tests subsequently effectively showed that the generated nanoparticles exhibit synergistic effects, with an increase in nanocomposite concentration inhibiting the growth of six fish bacterial strains. Aeromonas sobria, A. hydrophila, A. veronii, Vibrio cholera, Serratia fonticola, and Serratia odorifera were among these strains, in that order. Atomic Force Microscope (AFM) measurements showed that the generated nanocomposite particles had an average diameter of 52.63 nm, making them nanoscale. which lemon crude is successfully incorporated into films made of nanocellulose, it may exhibit antibacterial qualities against a range of bacterial species. Serratia fonticola, Serratia odorifera, Vibrio cholerae, Aeromonas sobria, A. hydrophila, and A. veronii. Given the efficacy of the technological technique of making their composites by loading them over prepared nanocellulose, the study's findings suggest that lemon crude can be utilized to treat bacterial illnesses in fish.

Keywords: Nano organic material, nanocellulose, Citrus limonum, Nanocomposites, Fish pathogens, Sustainable Green Treatment.

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INTRODUCTION

Over the past 20 years, a great deal of research has been done on the synthesis, stability, and toxicity of engineered metal nanoparticles (ENPs). On the other hand, only recently has interest turned to the study of naturally occurring nanoparticles' (NNPs') production, destiny, and ecological impacts (Sharma *et al.*, 2015). Consequently, there is a pressing need for sustainable and effective antibacterial agents in aquaculture. Nanocomposites have emerged as promising alternatives in this context. Among them, nanocellulose—a

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biopolymer derived from cellulose at the nanoscale-has garnered attention due to its biodegradability, high mechanical strength, and large surface area, which facilitates functionalization. Studies have shown that surface-functionalized nanocellulose exhibits notable antibacterial properties, making it a potential candidate for applications in food packaging and preservation (Klemm et al., 2011). Nanocellulose, a biodegradable and renewable nanomaterial derived from cellulose, has gained substantial attention in recent years due to its excellent mechanical properties, high surface area, and biocompatibility (Klemm et al., 2011). These attributes make nanocellulose a promising material for applications in food packaging, biomedical engineering, and aquaculture. In aquaculture, the prevention of bacterial infections is crucial, as microbial contamination in fish farming can result in significant economic losses and threaten food safety (Austin & Austin, 2007). The development of eco-friendly antibacterial agents is thus essential for sustainable aquaculture. One of the major drawbacks of nanocellulose is its lack of inherent antibacterial activity, limiting its application in microbial control. То overcome this limitation, surface modification strategies have been explored, aiming to impart antimicrobial functionality without compromising the material's biodegradability (Trache et al., 2017). A natural and promising approach involves the use of plant-based extracts rich in bioactive compounds. Citrus limon (lemon) is known for its high content of flavonoids, ascorbic acid, and essential oils such as limonene, which exhibit potent antibacterial activity against a wide range of pathogenic bacteria (Singh et al., 2020).

Incorporating lemon extract onto the surface of nanocellulose may create a synergistic material that combines the structural advantages of nanocellulose with the antimicrobial properties of lemon-derived compounds. This modification not only leverages natural and non-toxic agents but also aligns with the principles of green chemistry. Several studies have highlighted the antimicrobial efficacy of lemon extracts, particularly against common aquatic pathogens such as Aeromonas hydrophila and Vibrio species (Nayak *et al.*, 2019). However, there is limited research on the integration of lemon-derived antibacterial agents with nanocellulose, especially in the context of aquaculture and fish health.

Therefore, this study aims to develop and evaluate a novel antibacterial nanocomposite by modifying the surface of nanocellulose using lemon extract. The modified material will be assessed for its antibacterial properties, structural characteristics, and potential application in fish handling and packaging to reduce bacterial contamination and enhance shelf life.

MATERIAL AND METHODS

Isolation, Purification, and Identification of Bacteria In order to isolate microorganisms, intestines, skin, and gills (especially at lesion sites) were collected from fish as target organs using sterile swabs. Lit Bunsen burners and the inoculating swab loop are used to cultivate the samples. The lid of the Petri dish holding the nutrient agar medium is slightly ajar. The inoculating swab loop tip is then carefully pressed to the proper body area of the fish, and then TCBS, MaConckey, and nutritional agar are streaked over it. After being incubated at (37°C) in the inverted posture for (24 to 48 hours), the bacterial colonies were visually inspected and described. Morphological characteristics such as optical features, size, form, color, edge, and elevation were examined and recorded.

To confirm the findings of the motility test, a Vitek2 compact system was utilized. Furthermore, to identify the isolated bacterial which was extracted from common carp fishes, a gram staining reaction was included in the process (Kiriratnikom et al., 2000). The process kinetic analysis is based on test data that is fed to the Vitek2 device periodically within fifteen minutes. In order to measure colorimetric signals, turbidity, and fluorescence, a Multichannel fluorimeter and photometer values are integrated by the optical system to record (PINCUS, D.H. 2010). The identified bacteria with Vitek-2 compact system are actually extracted from various fish farms within the local area of Babylon city in Iraq. They include Aeromonas sobria (93%), A. hydrophila (95%), A. veronii (93%), Vibrio cholera (86%), (Hameed, Z.B, 2024).

Preparation of lemon Extracts

We gathered fresh Citrus limonum Burm fruits from the market. A plastic juice extractor was used to extract the juice from five lemon fruits after they had been peeled. Whatman Number One was used to doubly filter the extracted fruit juice. 0.45µm membrane filter (Sigma) and filter paper, respectively. At room temperature, the filtrate was evaporated to a paste after being measured into a sterile, weighted Petri dish. Afterward, the residue's weight was calculated and documented as the fruit juice extract's yield (w/v). For twenty hours, the fruit juice extract was sterilized by UV light. By plating an aliquot of the reconstituted extract on nutrient agar plates, its sterility was verified. Within two to four days of preparation, the extract was used for phytochemical analysis and antimicrobial susceptibility testing while being kept in sterile containers at 4° C.

Preparation of Nanocellulose

A measured weight of fresh potatoes has been obtained from After cleaning potato (peel removed) in distilled water. This sample was cut into small pieces with a knife cleaned in acetone or distilled deionized water, these pieces obtained as a homogenous shapes with (5 mm) per sample. By following this protocol, the possibility of tool contamination during sample preparation and collection was decreased (Hartman ,2006). Potatoes are crushed to obtain the extract, which is then stored in a dark container away from light and air to prevent oxidation. A piece of cloth was used to filter the sample after it had been mechanically squeezed to extract potato juice. Furthermore, the produced extract sample was protected from light or air oxidation by being placed in a labeled, dark container after the filtration phase, it can be dried using another kind of regular starch. heated for one hour at (50 °C) after combining starch with de-ionized distilled water to create a solution. To the extracted sample, three drops of lemon juice (citric acid) were added. . Centrifuge the powder for (10 min) at (6000 rpm) to isolate it, and then filter it through What Man filter paper 42 (2.5 µm). To obtain dry nanocellulose at room temperature, evaporate it and then wash it with de-ionized distilled water. The extracted organics' nanoproperties are then measured using the necessary techniques, including FTIR and AFM (Abed Al-Kadhim et al., 2022).

Preparation of Nanocomposites: Nanocellulose incorporating lemon

As previously reported, microcrystalline cellulose was used to create cellulose nanocrystals (CNC) utilizing environmentally friendly methods. (Abed Al-Kadhim et al., 2022). The following is a modified method for making natural novel composites (nanocellulose/lemon) in Tween 20 medium: More crude lemon was combined with various concentrations of Tween 20 (1%, 2%, 4%, 6%, 8%, 10%, v/v) while being stirred for 24 hours. Subsequently, 20,000 kHz sonication was applied for 30 minutes to the crude lemon solutions in Tween 20 media. A homogenous nanocomposite cannot be created without sonication. and an assortment of the supernatants was made. Subsequently, a crude lemon solution was combined with a varying quantity of nanocellulose in Tween 20 medium and agitated for approximately twenty-four hours.

Infrared Fourier-Transform Spectroscopy (FTIR)

The FTIR spectra of the extracted crude lemon, Tween 20, and crude lemon -loaded nanocellulose were recorded across the 4000–400 cm-1 range using the PerkinElmer Spectrum 400 FTIR instrument.

Analysis of Atomic Force Microscopy (AFM)

To examine cellulose nanoparticles, atomic force microscopy was employed. A few drops of the produced cellulose nanoparticles were applied to a silica glass plate to create a thin layer, which was then left to cure at room temperature in the dark. Following deposit, the film glass plate was scanned using the AFM (AFM,2019). The University of Baghdad's Department of Chemistry and College of Sciences provided the labs for this examination.

Antibacterial Activity

The composite of nanocelluloses with crude lemon extract was prepared in different concentrations. The bacterial suspension was screened for antibacterial activity using the agar-well diffusion method at an adjusted concentration equal to 0.5 McFarland standards. A sterile cotton bud was used to apply the bacterial solution evenly throughout the Muller-Hinton agar surface. Wells were made in agar plates containing inoculums using a sterile cork borer with a diameter of 6 mm. Each extract was then added to the appropriate well in the amount of 100 microliters. The plates were chilled for thirty minutes to guarantee that the extracts had completely penetrated the agar. After that, the plates were incubated at 37 °C for eighteen hours. To ascertain whether antibacterial activity was present, the zone of inhibition that formed throughout the incubation period was assessed (Valgas et al.,2007).

RESULTS AND DISCUSSION

Lemon and Nanocellulose Characterization:

1. Fourier Transform Infrared Spectroscopy (FT-IR):

It is clear from the FT-IR spectrum of lemon extract that it depends on three main regions of absorption bands of the frequency of chemical bonds of the active functional groups within the chemical structures present in the raw lemon extract. As is known in the scientific literature, it consists from mainly carboxylic acids such as citric acid and ascorbic acid to a large extent, and this is dominated by the appearance of carboxyl or carbonyl groups with acidic hydroxyl groups according to the **Fig. (1)**.



Figure 1: Structures of Citric acid and an Ascorbic acid

In **Figure (2)** of the crude lemon extract, there are three prominent peaks for the absorption of chemical bonds for the broad band of water hydroxyl (3425.34 cm⁻

¹), for carboxylic or carbonyl attached to hydroxyl groups (1731.98 cm⁻¹), and etheric group at (1215 cm⁻¹) respectively.



Figure 2: FT-IR spectrum of Lemon

While the **Figure (3)** that belong to nanocellulose which includes five main absorption peaks which are a broad band for water hydroxyl at $(3371.37 \text{ cm}^{-1})$, an aromatic C-H band at $(2927.94 \text{ cm}^{-1})$, an

absorption due to carbonyl group at (1649.14 cm⁻¹), an absorption of O-H and C-H groups at (1421.54 and 1369.46 cm⁻¹), and for etheric groups at range (1159.22 - 991.41 cm⁻¹) respectively.



Figure 3: FT-IR spectrum of Nanocellulose

In **Figure (4)** for prepared composite (lemon/ nanocellulose), which includes four main absorption groups, which are at (3434.98 cm⁻¹) for hydroxyl of water, an absorption of aromatic C-H group at (2925.8 cm⁻¹), as well as functional main absorption of carboxyl group at (1733.89 cm⁻¹) and an absorption for the ether group at (1105.14 cm⁻¹) respectively.



Figure 4: FT-IR spectrum of Nano Composite (Lemon/ nanocellulose)

2. Atomic Force Microscopy (AFM) Measurements 1. Nanocellulose

Atomic force microscopy (AFM) measurements of nanoscale of prepared nanocellulose from lab starch for the purpose of identify their effectiveness and physicochemical behavior, was confirmed by showing the mean diameter (84.00 nm) and surface area (11966 nm²) as in figure (6), but with

significant nature and clear homogenous surface roughness, as observed in the surface images of nanocellulose as in figure (5). This is due to nature of nanocellulose crystals and their chemical structure.

Whereas, an arithmetic mean height was (60.23 nm) according to topology parameters of surface roughness as in figure (7).



Figure 5: Surface Images of Nanocellulose



Figure 6: Particle analysis – Threshold detection of nanocellulose

From the above figure, analysis of nanocellulose particles through the homogeneity shown by histogram for 3D view of the surface, mean diameter

scheme, and high surface area, which clearly shows the nanoscale of the prepared nanocellulose.



Figure 7: Roughness analysis of nanocellulose

In **Figure (7)**, the surface roughness analysis of the prepared nanocellulose is shown, which shows five main parameters: height parameters, functional parameters, spatial parameters, hybrid parameters, functional parameters (volume), and feature parameters related to the nature, shape, roughness, and homogeneity of the surface of the nanocellulose particles.

2. Nano Composite of Lemon/ Nanocellulose

Atomic force microscopy measurements of nanoscale of the prepared Nano composite (lemon/ nanocellulose) for the purpose of identify their effectiveness and physicochemical behavior, was confirmed by showing the decreasing of mean diameter (52.63 nm) and surface area (5511 nm²) as in **figure (9**),

but with significant nature and clear more homogenous surface roughness than nanocellulose, as observed in the surface images of Nano composite as in **figure (8)**.

This is due to the role of lemon extract added to the prepared nanocellulose in enhancing the acidity, improving the smallness of required nano-property of prepared Nano composite, and breaking the beta linkages among building blocks of nanocellulose, which was prepared mainly by the acid hydrolysis method of lab starch.

Whereas, an arithmetic mean height was (30.64 nm) according to topology parameters of surface roughness as in **figure (10)**.



Figure 8: Surface Images of Nano Composite (Lemon/Nanocellulose)



From the above figure, analysis of prepared particles of Nano composite through the high homogeneity shown by histogram for 3D view of the

surface, mean diameter scheme, and surface area, which clearly shows the high preferred nanoscale of the prepared composite.



Figure 10: Roughness analysis of Nano Composite (Lemon/nanocellulose)

In Figure (10), the surface roughness analysis of the prepared Nano composite of lemon over nanocellulose is shown, which shows five main parameters: height parameters, functional parameters, spatial parameters, hybrid parameters, functional parameters (volume), and feature parameters related to the nature, shape, roughness, and high homogeneity of surface of the prepared Nano composite.

From the particle and roughness analyses and surface images in **figures (5-10)** of the nanocellulose and their Nano composite with lemon, the difference was evident for the surface of prepared Nano composite (lemon/nanocellulose), as it was characterized by the plenty of sharp dense peaks of low various sizes, which led to an increase in the values of Kurtosis and Skewness as a height parameters compared to the nature of the surface of the nanocellulose alone.

In order to gain some useful information about the height, functional, and hybrid of topology parameters regarding the prepared Nano surface of organic green materials such as nanocellulose and their Nano composite (Lemon/ Nanocellulose) as described in **Table** (1).

Parameters	Nanocellulose	Nano Composite (Lemon/ Nanocellulose)
Root-mean-square height (nm)	81.29	57.44
Skewness	0.2043	7.724
Kurtosis	3.905	99.98
Arithmetic mean height (nm)	60.23	30.64
Inverse areal material ratio (nm)	101.2	40.56
Material ratio height difference (nm)	212.3	90.78
Developed interfacial area ratio	7.243	24.07

Table 1: Surface roughness analysis from AFM measurements

These properties and nano-parameters of the surface topology of prepared Nano composite (lemon/ nanocellulose) which is the required useful tool with low nanoscale, large surface area, good crystallization, biodegradability, and application in various vital processes such as nutrition, adsorption or encapsulation of medicines, treatment of polluted water, inhibition of bacteria, decomposition of toxic organic compounds, sensors for pathogens in water, absorbents for oil spills in water and sequestering carbon dioxide in the atmosphere, nanocellulose-based membranes which are used to remove various heavy metals, dyes, oils and pharmaceuticals that still pose a threat to aquatic life and thus for human and environment (Sujjad Hadi and Alaa Al-Khalaf, MSc thesis, 2022).

A cellulose nanomaterial with unique physicochemical properties has been applied as modern multifunctional smart tool in many vital fields, (Al-Jawasim, M., Al-Khalaf, A., 2022).

Antimicrobial Assay

The agar-well diffusion method results demonstrated varying degrees of growth inhibition in the nanocomposites crude lemon extract made with nanocellulose, as detailed in the Materials and Methods section. The nanocellulose-based lemon showed notable antibacterial activity against Gram-negative The concept of combining antimicrobial agents is that these mixtures can enhance their effectiveness, reduce toxicity, minimize side effects, increase how well they work in the body, require smaller amounts, and slow down the development of resistance to antibiotics(Ching *et al.*, 2019).Research priorities have emerged for new antibacterial combination medicines that incorporate mixtures of natural products (Van Vuuren and Viljoen, 2011). Nanocellulose can be chemically modified to enhance its surface properties, allowing for the effective incorporation of lemon extracts (Zhang *et al.*, 2023).

Antibiotics to prepare lemon crude-loaded nanocellulose in Tween 20 medium statistically showed greater antibacterial activity against. Aeromonas sobria, A. hydrophila, A. veronii, and Vibrio cholerae. Serratia fonticola. The binary nanocomposite (nanocellulosebased lemon) demonstrated its high antibacterial activity against the six species of isolation bacteria, as they had a maximum mean value of inhibition zone diameter (22 mm) in bacteria Aeromonas soberia, as in Figure 11, while the minimum mean value of inhibition zone diameter (5 mm) was in bacteria Serratia odorifera, as in Figure 12. His binary nanocomposite (nanocellulosebased lemon) also has high antibacterial activity against four other types of bacteria. Their mean inhibition zone values reached 20 mm in bacteria Aremonas veronii, as in Figure 13, and 18 mm in bacteria Serratia fonticola, while the zone of inhibition was 12 mm in diameter for the type of bacteria Vibrio cholerae, and the zone of inhibition value was 10 mm for the type of bacteria Aremonas hydrophila, at the binary nanocomposite (nanocellulose-based lemon). The reason (s) for the disparities in the activity of the nanocomposite in this work and several others othersremain unexplained. Although differences in the diffusion rate of the antimicrobial agent in the agar have been tendered as an explanation for such disparities, it is a phenomenon that needs further investigation in order to achieve acceptable standards for methods. The antibacterial activity of lemon fruit juice may be attributed to the presence of organic acids, constituent vitamins, secondary metabolites, and their interactions with each other. Citric acid, but not malic acid, has been shown to contribute to the antibacterial activity of Citrus sudachi juice. Further investigation is required to identify the active ingredient(s) that either singly or in combination account for the antibacterial properties of C. limonum fruit juice.



Figure 11: Mueller–Hinton agar plates showing the binary nanocomposite (nanocellulose-based lemon) maximum mean value of inhibition zone diameter in Aeromonas soberia



Figure 12: Mueller–Hinton agar plates showing the binary nanocomposite (nanocellulose-based lemon) minimum mean value of inhibition zone diameter in Serratia odorifera



Figure 13: Mueller–Hinton agar plates showing the binary nanocomposite (nanocellulose-based lemon) mean value of inhibition zone diameter in Aremonas veronii

Overall, the antimicrobial activity of the lemon fruit juice extract against know etiologic agents of typhoid fever, gastroenteritis and wound sepsis validates the folkloric claims of its efficacy in the treatment of these and other bacterial infections (Kafaru, 1994) Lemon extracts contain compounds such as citric acid and flavonoids, which exhibit significant antibacterial activity against various pathogens, including those affecting fish These compounds can disrupt bacterial cell membranes, leading to cell lysis and death, making them effective against fish pathogens like Aeromonas salmonicida (Furushita et al., 2003). Techniques such as grafting and coating can be employed to attach lemonderived antibacterial agents to the nanocellulose surface. improving its functionality without compromising its biocompatibility (Dannong et al., 2019). Recently, chemical modification of cellulose to obtain antibacterial materials with non-leaching property and prolonged biocidal activity has gained lots of attention (Feese et al., 2011) (Fernandes et al., 2013). Furthermore, low pH is fatal to a broad spectrum of bacteria and although there are some bacteria that can survive in acidic environments (Cotter and Hill ,2003) Because cellulose has a lot of reactive groups (hydroxyl groups) on its surface, it may easily be functionalized with other functional groups, including amines, carboxylic acids, and aldehydes, to produce a variety of characteristics. Furthermore, the variety of substances that can be grafted onto cellulose's polymers, including proteins, structure, metal nanoparticles, and antibiotics, is increased by the material's ease of surface modification. From nanocomposite' antibacterial action, microbial cell death is brought on by altered membrane permeability, decreased nucleic acid production, and chemical alterations in microbial organelles (Liu, X. et al., 2020) A large quantity of ammonium ions released into the culture media may have been the cause of the early antibacterial activity observed in the antibacterial tests (Luque-Alcaraz, A. G et al., 2016). The modified nanocellulose can be utilized in aquaculture settings, potentially as coatings for fish feed or as part of packaging materials, thereby reducing bacterial load and enhancing fish health (Ying et al., 2015). This approach not only addresses the immediate need for antibacterial solutions but also aligns with sustainable practices in aquaculture. While the integration of lemon extracts into nanocellulose shows great potential, further research is necessary to fully understand the mechanisms of action and optimize the effectiveness of these modifications in real-world applications.

CONCLUSION

Despite the impressive progress made in the creation of antibacterial materials, a number of problems still need to be addressed in order to provide biocompatible and biodegradable surfaces with durable antibacterial properties and reasonably cheap costs.

These problems could be greatly resolved by cellulose, the most prevalent biopolymer on the planet.

Although cellulose in its pure state lacks inherent antibacterial qualities, it is rich in functional groups that can be used in a variety of ways. For instance, they can be directly used to attach natural antibacterial agents to make cellulosic antibacterial compounds like The C. limonum fruit juice extract showed broad-spectrum activity similar to Gentamycin against the investigated strains of bacteria.

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