SYNTHESIS, CHARACTERIZATION AND EVALUATION OF ANTIBACTERIAL PROPERTIES OF ALOE VERA / CHONDROITIN SULFATE / NANOCELLULOSE NANOCOMPOSITE HYDROGEL

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Abstract

Authors report the synthesis of nanocomposite hydrogel as an antibacterial agent. In this work, the nanocomposite hydrogel was synthesized using freeze-drying method by blending Aloe vera, chondroitin sulfate and nanocellulose. The synthesized nanocomposite was characterized using Fourier transform infrared (FT–IR), scanning electron microscopy (SEM) and thermogravimetric analysis (TGA). The evidence showed that there is a noteworthy chemical interaction between the Aloe vera, chondroitin sulfate and nanocellulose. In addition, equilibrium degree of swelling of nanocomposite hydrogel was determined gravimetrically. The antibacterial studies of Aloe vera / chondroitin sulfate / nanocellulose nanocomposite hydrogel was evaluated against Escherichia coli (gram-negative) and Staphylococcus aureus (gram-positive) bacteria. The results illustrated that the nanocomposite hydrogel has good antibacterial activity against gram-positive and gram-negative bacteria and can be suitable for antimicrobial applications.

1. Introduction

Hydrogels have been defined in many different ways by researchers over the years. The most usual of these is that hydrogel is a water-swollen, and cross-linked polymer chains produced by the simple reaction of one or a few monomer units. Another definition is that hydrogel is a hydrophilic polymer that shows the ability to swell and retain amount of water within its structure, but will not dissolve in water. Hydrogels have gained significant attention in the past 50 years, due to their special applications **[1, 2]**. Hydrogel-based products constitute a group of polymeric materials, the hydrophilic structure of which would make them capable of holding vast amounts of water in their three-dimensional networks. Using of these products in a number of environmental and industrial areas of application has a great importance **[2]**.

Hydrogel polymers are three-dimensional hydrophilic compounds that are able to absorb, expand, and hold large amounts of liquid [3, 4]. Polymeric hydrogels have empty pores / spaces between their cross-linked tissues. Therefore they can be used to synthesize nanoparticles and act as nanoreactors [5]. Among the various polymers proposed for the preparation of hydrogels, polysaccharides have many practical advantages over the synthetic polymers initially employed in the field of pharmaceutics [6]. Polysaccharides contain more than ten monosaccharide units linked by glycosidic bonds. Besides, polysaccharides can be obtained from various abundant renewable resources in nature, namely plant (cellulose, acemannan, and starch), algae (e.g. agarose, alginate, carrageenan, fucoidan, and ulvan), microbial (bacterial cellulose, dextran, and gellan gum) and animals (e.g. chondroitin sulphate, chitin / chitosan, glycosaminoglycans, hyaluronan, and heparin) [7, 8]. Polysaccharides, as natural biopolymers, are biocompatible, biodegradable and non-toxic. Polysaccharides have a great number of functional groups such as carboxyl, hydroxyl and amino groups that make them easily physically blended or chemically modified [9, 10]. These characteristics together with their inherent and biological properties proposed that polysaccharides are promising biomaterials. Hydrogels containing polysaccharides have biocompatibility with the human body. Therefore they have been widely used in biomedical applications. In addition, hydrogels are similar to natural living tissue more than any other category of synthetic biomaterials, because they have high water content and soft consistency, which makes them resemble natural tissues [11].

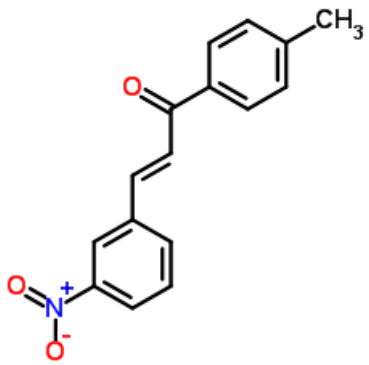

Figure 1. Schematic representation of Aloe vera leaf pulp structure and its components.

Aloe vera (AV) is a natural tissue used to produce hydrogel. The Aloe vera plant is a member of lily family which is full of juice and similar to a cactus. Aloe vera pulp consisted of three structural components including the cell walls, the degenerated organelles and the viscous liquid contained within the cells. These three components are distinctive from each other both in terms of sugar composition and morphology [12] as shown in **Figure 1**. Many reports have suggested that Aloe vera gel has numerous beneficial properties for wound healing, including the abilities to penetrate and anesthetize tissue, prevent bacterial, viral and fungal growth, act as an anti-inflammatory agent and enhance blood flow [13]. Furthermore, Aloe vera has been used externally to treat kind of skin conditions such as eczema, cuts and burns. It is supposed that sap from Aloe vera reduces inflammation and pain. It has antibiotic and antiseptic characteristics which make it highly valuable in treating abrasions and cuts [14, 15]. *In vitro* extracts of Aloe vera motivate the proliferation of several cell types. Many studies have shown that treatment with whole Aloe vera gel extracts resulted in faster healing of wounds [16, 17].

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The researchers have confirmed the effect of Aloe vera on increasing collagen synthesis and wound contraction. This property is ascribed to the mannose-6-phosphate known to be present in Aloe vera gel [**18**]. Polysaccharides from Aloe increase both the proliferation of fibroblasts and the production of hydroxyproline and hyaluronic acid in fibroblasts, which play significant roles in extracellular matrix remodeling within wound healing [**19**].

Chondroitin sulfate (CS) is a sulfated glycosaminoglycan that is usually found attached to proteins as part of a proteoglycan. CS is a biopolymer which has different advantages such as biocompatibility, biodegradability, availability, and highly versatility. CS is composed of a chain of alternating disaccharide unit polymers of D-glucuronic acid and N-acetyl galactosamine sulfated at either 4- or 6-positions [**20**]. Chemical structure of one unit in a CS chain [**21**] is shown in **Figure 2**. CS is found in natural cartilage and other tissues, and has biological properties including degradable, non-toxic, adhesive, anti-inflammatory, thickening, promoting cartilage regeneration, and good biocompatibility [**22**].

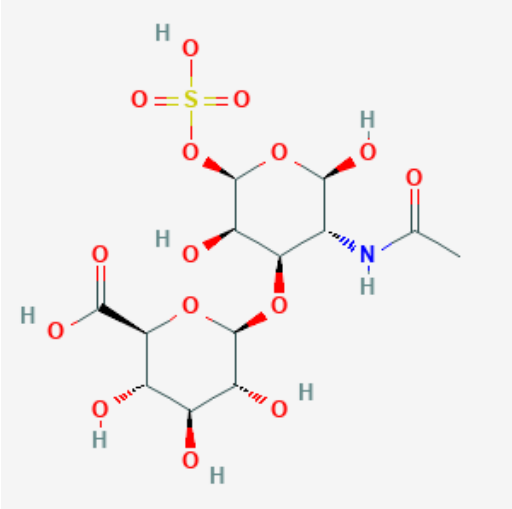

Figure 2. Chemical structure of one unit in chondroitin sulfate chain. Chondroitin-4-sulfate: R_1 = H, R_2 = SO_3H, R_3 = H. Chondroitin-6-sulfate: R_1 = SO_3H, R_2, R_3 = H.

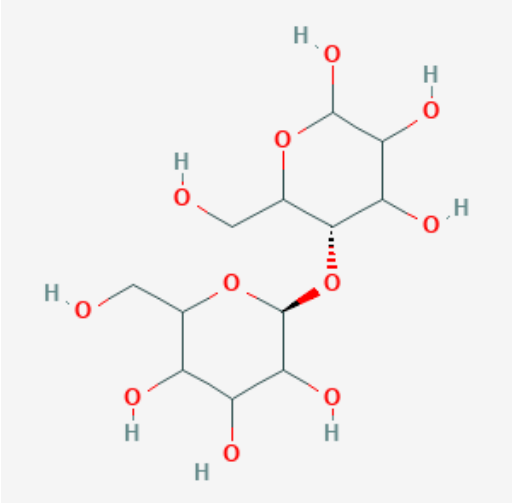

Figure 3. Chemical structure of cellulose which is linear polymer made up of β-D-glucopyranose units covalently linked with (1-4) glycosidic bonds.

Cellulose, as the main component of the plant cell wall, is a glucose polymer. Cellulose is not water soluble, but it has many hydroxyl groups which cause to form strong hydrogen bands **[23]**. Chemical structure of cellulose is shown in **Figure 3**. Nanocellulose (NC) has a high surface/volume ratio, activity and crystallization ability. Nanocellulose has many advantageous physical, chemical, and biological characteristics. It has good stability against proteolytic enzymes, acids, and temperatures, and high biodegradability **[24]**. Nanocellulose-based materials in general have high physical, chemical, electrical, thermal, and optical properties. Moreover they have chemical inertness, mechanical strength, barrier properties, tailor able morphological features. Their biocompatibility with no toxicity or low toxicity and with low immunogenicity and their antimicrobial effects are amazing. In addition they are available, renewable and relatively low-cost materials **[25]**. Among their properties, nanocellulose-based materials have received much attention for antibacterial application because of their remarkable physical properties, special surface chemistry, and excellent biological properties **[26]**.

Nanomaterials with inherent antimicrobial activity or nanomaterials that can improve the efficacy and safety of antimicrobial drugs are called nanoantimicrobials (NAMs). A set of inorganic, organic, and hybrid materials can be considered in the NAM family **[27]**. Among all the NAMs, hydrogel is a 3-dimensional network of cross-linked polymer that can swell dramatically in the presence of an aqueous medium such as body fluids, while maintaining its structure and controlling drug release **[28 – 30]**. Antimicrobial hydrogels can be coated on central venous catheters, urinary catheters **[32]**, joint and dental implants, contact lenses **[32, 33]**, and local injection for drug release and wound healing **[34]**, Moreover, some kinds of hydrogels also have inherent antimicrobial characteristics **[35, 36]**. These properties have gained considerable attention in the medical and pharmaceutical fields especially for antimicrobial application.

2. Materials and methods

2.1. Materials

The materials used were nano cellulose powder (Nanosadra), chondroitin sulfate sodium salt (Sigma-Aldrich), Aloe vera (Generic), Glutaraldehyde 25 % (Acros) as a cross-linker aget, distilled water was used throughout this study.

2.2. Preparation of Aloe vera / chondroitin sulfate / nanocellulose hydrogel

Hydrogel sample was prepared using mechanical stirrer through cross-linking. 4 g Aloe vera was initially dissolved in distilled water. 3 g nanocellulose powder was added to the solution and stirred until homogenous mixture appeared. Then 0.2 g chondroitin sulfate was added to the Aloe vera / nanocellulose mixture, under vigorous and continuous stirring. Subsequently, suitable amount of glutar aldehyde 25 % was carefully added to the mixture of hydrogel with continuous stirring as a cross-linker agent until a homogenous mixture obtained.

Obtained hydrogel was undergone to freeze drying for 24 h by means of a freeze dryer VaCO5 by Zirbus (German) company.

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2.3. Characterization

2.3.1. FT–IR spectroscopy

The Aloe vera, NC, CS and obtained hydrogel samples were analyzed by FT–IR spectroscopy, using the KBr pellet technique. The spectra were scanned on a Jasco (Japan) device, over the 4000 – 400 cm^{-1} range at a resolution of 4 cm^{-1}.

2.3.2. Scanning electron microscopy (SEM)

After metallization with gold, the surface morphology of prepared hydrogel was investigated using a scanning electron microscope (FESEM, Quanta 450 FEG – FEI, USA). Magnification is given on pictures.

2.3.3. Thermogravimetric analysis (TGA)

Thermogravimetric analysis was performed using Labsys Evo TGA by Setaram. The profile of sample was recorded from 25 to 750 °C, under argon atmosphere. The thermal stability of the hydrogel matrix was studied using the thermogravimetric data.

2.3.4. Swelling studies

Equilibrium degree of swelling (EDS) was determined gravimetrically. The hydrogel samples dried to constant weight were immersed in the water at room temperature, for ~ 24 h. The excess water was removed with a filter paper and the samples were weighed. The EDS % was calculated using the equation: $EDS\ \% = (W_e - W_d) \times 100\ \% / W_d$, where, W_e is the weight of the swollen hydrogel at equilibrium and W_d is the initial weight of the dried hydrogel.

2.3.5. Antibacterial activity

The antibacterial assays were done against gram-negative Escherichia coli (E. coli) and gram-positive Staphylococcus aureus (S. aureus) bacteria by disk diffusion. The agar medium was used to cultivate bacteria.

3. Results and discussion

3.1. FT–IR spectroscopy

Figures 4a–d show the FT–IR spectra of the NC, AV, CS and NC / AV / CS nanocomposite hydrogel, respectively. In **Figure 4a**, the broad band at 3600 – 3000 cm^{-1} corresponds to the characteristic –OH stretching from vibrations in the intra- and intermolecular hydrogen-bonded hydroxyl groups in cellulose I. The band at 2901 cm^{-1} associated with aliphatic saturated CH-stretching in the glucose units. Other peaks detected contain the adsorption band at 1640 cm^{-1} which is related to water absorbed onto cellulose. The peak at 1430 cm^{-1} associated with CH_2 scissoring motion and symmetrical bending in cellulose I. There are found the peak at 1164 cm^{-1} corresponding to the asymmetrical bridge C–O–C stretching from the glycosidic bond, the band at 1112 cm^{-1} representing stretching of the glucopyranose unit, and the peak at 898 cm^{-1} typical of the β-glycosidic linkage in cellulose I **[23]**. FT–IR spectra of the AV (**Figure 4b**) shows several functional groups that represents three centered main peaks at 572, 1635, and 3454 cm^{-1} and five weak peaks at 1041, 1251, 1402, 2065, and 2351 cm^{-1}. The widest observed peak at wave numbers of 2900 – 3600 cm^{-1} was attributed to the stretching vibration of O–H. Furthermore, a minor centered peak at 1402 cm^{-1} could be related to the existence of carboxylic components. The centered peak at 1635 cm^{-1} was attributed to the amide I group **[24]**.

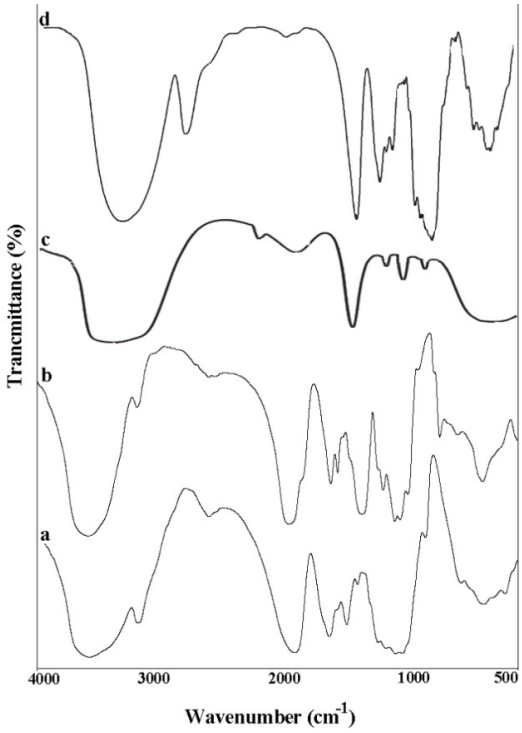

Figure 4. FT–IR spectra of: (a) CNC, (b) AV, (c) CS, and (d) CNC / AV / CS nanocomposite hydrogel.

FT–IR spectra of CS (**Figure 4c**) showed the characteristic peaks of absorption at the wave numbers of 1236, 1420 and 1635 cm^{-1} indicating the presence of sulfate, amine, and carboxyl groups. The characteristic equatorial bending vibrations peaks of C–O–S was observed at 825 and 875 cm^{-1} **[25]**. In **Figure 4d**, the OH absorption of NC, AV and CS in NC / AV / CS nanocomposite hydrogel can be observed at 3396 cm^{-1}, which becomes wider than that of NC, AV and CS themselves. So the characteristic absorptions of COOH and OH in NC / AV / CS nanocomposite hydrogel suggested the formation of hydrogen bond between NC, AV, and CS. That means the existence of much stronger interaction between NC, AV, and CS in NC / AV / CS nanocomposite hydrogel. The absorption bands at 1635 cm^{-1} could be characteristic of the amide of CS in NC / AV / CS nanocomposite hydrogel. In addition, the band at 1628 cm^{-1} corresponded to the absorption of OH group of NC, which was seen as a sharper peak at 1635 cm^{-1} in NC / AV / CS nanocomposite hydrogel may be due to the overlap of the bands of OH group in NC and amide in CS **[26]**.

3.2. Scanning electron microscopy (SEM)

The morphology of the resulting samples was investigated by scanning electron microscopy. The pure cellulose nanocrystal shows an entangled network of fibrils that can be seen in **Figure 5a**. In **Figure 5b**, it was shown that the layer of Aloe vera gel consists of organic fibers and particles with crushed and irregular shapes. This may be because of the degradation of the materials during the time **[27]**. The pure CS illustrated a macro porous structure similar to grape clusters (**Figure 5c**). Forming networks is visible in the nanocomposite hydrogel (**Figure 5d**). The lattice walls are rough in the nanocomposite hydrogel. It is due to the interaction between the polymer chains.

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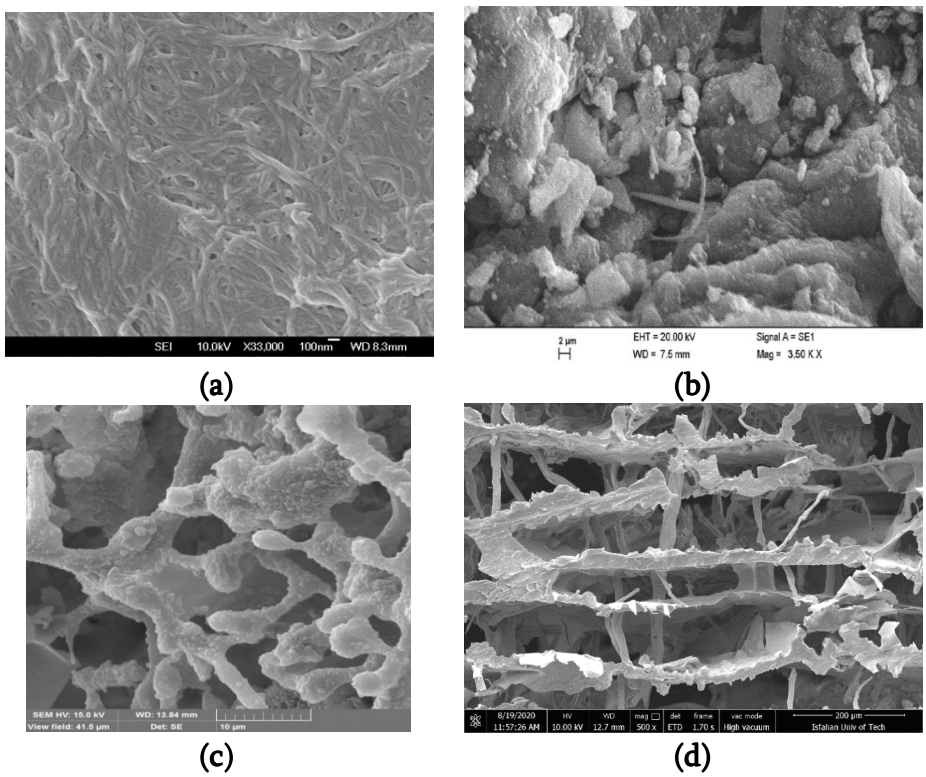

Figure 5. SEM images of: **(a)** CNC, **(b)** AV, **(c)** CS, and **(d)** nanocomposite hydrogel.

3.3. Thermogravimetric analysis (TGA)

TGA was carried out to evaluate the thermal behavior and compositional fraction of the nanocomposite hydrogel. TGA curves of pure CS and NC / AV / CS nanocomposite hydrogel are represented in **Figure 6**. TGA curve of pure CS particles shows a continuous weight loss of 9.5 % that is attributed to loss of moisture. The initial weight loss which observed around 100 °C can be related to water evaporation. There is a decrease after 195 – 385 °C that is obvious in the figure. The thermal decomposition of the organic component in NC / AV / CS nanocomposite hydrogel performed mostly in the range of 195 – 315 °C. Thermal analysis showed that NC and AV molecules have been interacted with CS that can be affected on rate of decomposing.

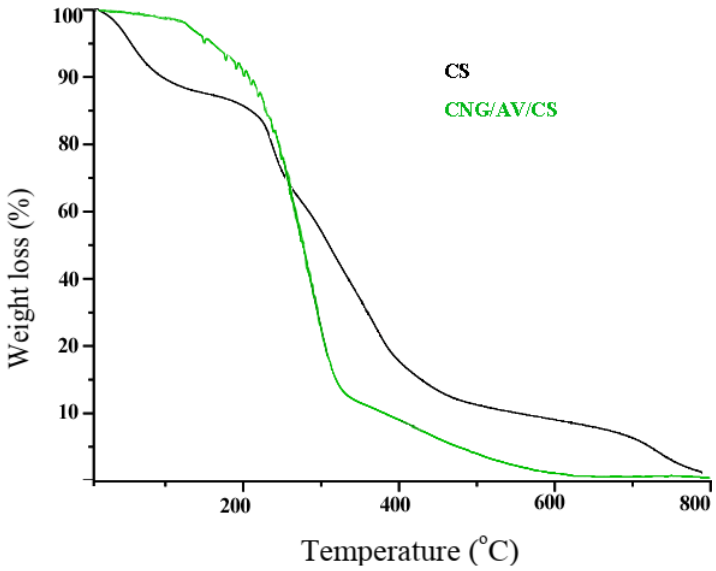

Figure 6. TGA curves of: (a) pure CS and (b) CNC / AV / CS nanocomposite hydrogel.

3.4. Swelling studies

The chemical composition of the hydrogel components has impact on the swelling ratio of the matrices. In the hydrogels system, absorption of solvent from the environment affects the physical-chemical properties and dimensions of the pores of the system. The large quantities of water attract and fast swelling in NC / AV / CS nanocomposite hydrogel can be related to the existence of negatively charged functional groups in chondroitin sulfate structure, such as –SO_3– and COO–, which cause to swell the gel, producing a high concentration of negative charge in the regions that contain them. The presence of ionization groups on CS chain causes the strong repulsion of negative charges and polar groups so the network chain segments apart and thus more water uptake into the hydrogels, so a higher swelling ratio was observed **[28]**. Also, Aloe vera can significantly increase water absorption. This behavior can be attributed to the hydrophilic properties of Aloe vera, which may improve the hydrophilicity of the hydrogel and enhance the affinity with water **[29]**. The EDS % was calculated using the above equation: EDS % = (8.36 – 0.61) × 100 % / 0.61 = 1270 %.

The freeze-dried hydrogel showed an extensive and fast swelling which can be explained by the morphological analysis (**Figures 5a–d**). As the hydrogel has interconnected pores, these channel-like structures can attract the water phase in the matrix through capillary action, and result in swelling by fast diffusion of solvent in the matrix. Morphological characterization helps to explain **[30]** hydrogel properties like hydrophilic nature, swelling behavior, etc.

3.5. Antibacterial activity

The antibacterial activity evaluation performed on the nanocomposite hydrogel to determine the ability of the nanocomposite hydrogel to inhibit bacterial growth. The percentage of bacterial cell death describes the proportion of bacterial cells that can be killed in 24 h compared to positive and negative control. The negative control kills 0 % of bacterial cells while the positive control kills 100 % of bacterial cells for both gram-positive and gram-negative bacteria. The *in vitro* antibacterial properties of hydrogel were assayed against gram-positive S. aureus and gram-negative E. coli bacteria by disk diffusion technique.

S. aureus, a major bacterium of the Micrococcaceae family, is part of the normal flora of human mucous and skin. It is able to colonize asymptomatically nearly a third of the population **[31]**. In the last two decades, the researchers have introduced S. aureus as a major etiological factor of soft tissue and skin infections. S. aureus is appeared as the most common etiological factor of hospital-acquired (nosocomial) skin infections and the most common cause of infection in surgical incisions. It makes critical systemic infections in humans **[32]**. Fast adaptation of S. aureus to environmental changes leads to impressive spreading in the environment, infecting other patients in hospitals and the population outside hospitals.

Escherichia coli also known as E. coli is a coli form bacterium, rod-shaped, gram-negative, and of the genus Escherichia that is generally lives in the lower intestine of warm-blooded organisms (endotherms). Some species can cause some diseases such as diarrhea, fever, vomiting and abdominal pain in humans **[33]**.

Figure 7 exhibits the typical antibacterial test results of hydrogel sample against (a) gram-negative E. coli and (b) gram-positive S. aureus by the disk diffusion method which

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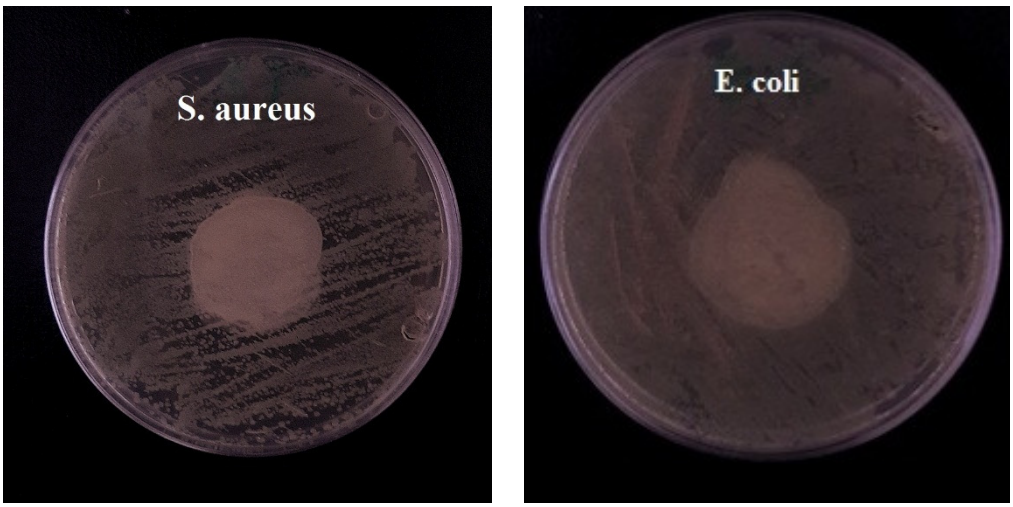
antibacterial activity is measured by the diameter of the inhibition zone under and around the tested samples. In this test, firstly, the tested bacteria were cultivated on the surface of nutrient agar plates, and then the hydrogel sample was placed on the surface. After 24 h in 37 °C incubation, no S. aureus and E. coli bacteria colonies could grow around hydrogel sample on the agar plate.

The inhibition zone recorded for hydrogel sample against gram-negative (E. coli) and gram-positive (S. aureus) were 13 and 10 mm respectively. Our results show that the hydrogel is more effective towards the gram-negative bacteria than the gram-positive bacteria strains. This may be because of the differences in the structure of the bacterial cell wall organization. In addition to the fact that gram-positive bacteria have a thicker layer cell than gram-negative bacteria, it acts as a barrier and protects the cell wall from the expansion of active component into the cytoplasm **[34]**.



(a) **(b)**

Figure 7. Antibacterial activity of hydrogel sample against **(a)** gram-positive S. aureus and **(b)** gram-negative E. coli by disk diffusion method.

Antimicrobials can inhibit the growth of bacteria at low concentrations and kill bacteria at high concentrations. The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial ingredient that is bacteriostatic or prevents the growth of bacteria. The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill bacteria. In our work, inhibition of E. coli bacterial growth happened at concentration of 6000 ppm. For S. aureus, 100 % inhibition was achieved at 8000 ppm. The MIC value for S. aureus was greater than that for E. coli, indicating that S. aureus is more resistant to hydrogel as an antibacterial factor **[35]**.

Our results showed that the nanocomposite hydrogel has good antibacterial activity against gram-positive and gram-negative microorganisms, using E. coli and S. aureus as models. This antibacterial activity of hydrogel is attributed the antibacterial activity of each hydrogel components.

Aloe vera has anthraquinone which is an active compound and structural analogue of tetracycline. The anthraquinone acts like tetracycline that prevents synthesis of bacterial protein by obstruction the ribosomal A site (where the aminoacylated RNA enters). Therefore, the bacteria cannot grow in the media including Aloe vera extract **[36]**. Direct bacterial activity via the motivation of phagocytic leucocytes to destroy bacteria have been ascribed to

polysaccharides of Aloe vera gel. Also, Aloe vera contains pyrocatechol as a hydroxylated phenol, which is known to have toxic effect on microorganisms [**37**].

Another component of hydrogel sample, chondroitin sulfate (a natural-origin saccharide), is a noteworthy option as antibacterial agent, because the carbohydrate moieties can cause both biocompatibility and hydrophilic features to the material surface [**38**].

In addition to antibacterial properties of Aloe vera and chondroitin sulfate, the third component of the hydrogel, nanocellulose, have gained much attention for antibacterial application because of their remarkable physical properties, special surface chemistry, and excellent biological characteristics [**37, 39**].

4. Conclusion

Aloe vera / chondroitin sulfate / nanocellulose hydrogel was successfully synthesized in this study using the physical-chemical method. Characterization using FT–IR, SEM and TGA techniques showed that the nanocomposite hydrogel synthesized successfully. Swelling studies showed that the freeze-dried hydrogel showed a fast and extensive swelling.

In this study, the observed hydrogel MIC values against E. coli and S. aureus were 6000 and 8000 ppm, respectively. The results showed that the nanocomposite hydrogel has good antibacterial activity against gram-positive and gram-negative bacteria.

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