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The effect of impregnated alpha-cellulose nanofibers with ciprofloxacin hydrochloride on *Staphylococcus aureus in vitro*

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The main objective of this analytical and descriptive study is to evaluate the effect of alpha-cellulose nanofibers impregnated with ciprofloxacin hydrochloride on a staphylococcus aureus culture. In this regard, after conducting various studies on biocompatible and natural polymers, wheat bran was selected as a cellulosic-based biocompatible agricultural waste. After alpha-cellulose extraction from wheat barn by the ultrasonic-Soxhlet method. the alpha-cellulose investigated with FTIR. FTIR spectra showed that holocellulose was largely removed during alkali treatment under ultrasonic waves. the alpha-cellulose nanofibers were prepared by employing the electrospinning technique. from which the cellulose disks were prepared. Then, these disks and paper disks (without antibiotics) were placed in 3.3% ciprofloxacin hydrochloride. The mentioned disks were placed on the Staphylococcus aureus cultured medium alongside standard disks and the results were measured as the inhibition zone after 24 hours. Both disks (alpha-cellulose nanofibers and paper) containing ciprofloxacin hydrochloride created the inhibition zone on the S. aureus medium. The concentration of ciprofloxacin hydrochloride adsorbed by cellulose was determined by comparing the effects of cellulose disks containing different concentrations of antibiotics and standard ciprofloxacin disks.

Keywords: ciprofloxacin hydrochloride, disk diffusion, alpha cellulose nanofiber, electrospinning, in-vitro.

INTRODUCTION

In comparison to conventional nanofiber production methods, electrospinning has been introduced as a simple, rapid, efficient, and inexpensive technique to produce nanofibers. The controlled fibrous structures can be successfully produced for application in similar biological structures by controlling the effective electrospinning parameters. The nanofibers obtained by this method can be employed in medicine, controlled drug release, tissue engineering, wound dressing, and filtering. Based on their origin, polymeric wound dressings have been generally classified as synthetic polymers, natural polymers, or their combination. The synthetic dressings include water vapor-permeable films, hydrogels, hydrocolloids, alginates, and antibacterial dressings [1, 2, 3, and 4]. The film dressings have a poor ability to absorb wound secretions due to their accumulation under the dressing; thus, they are not suitable for highly secreting wounds. Hydrogel-, alginate-, and hydrocolloid-based dressings are able to absorb wound secretions. Conventional hydrogel-based dressings, however, have low mechanical strength and do not provide adequate wound protection against mechanical stresses [5 and 6]. Hydrocolloids have lower water vapor permeability, thus lacking the required properties as a wound dressing. Moreover, hydrocolloids components may provoke skin sensitization [7, 8, and 9]. Alginate dressings are suitable for ulcers with moderate to high grades of secretion; however, they lack intrinsic antimicrobial properties. Collagen and chitosan are among the natural polymers that are used in wound dressings. The advantage of natural polymers is that they can accelerate the healing process [10, 11, and 12].

One of the problems with these structures is their high cost due to their limited availability. Therefore, their integration with synthetic polymers could be effective in cost-reducing strategies. Antimicrobial dressings are new structures that eliminate the need for frequent sterilization and dressing change, thus saving on treatment costs [13, 14, and 15]. Numerous attempts have been devoted to the construction of chitosan fibers through electrospinning, which did not succeed except in a few cases as chitosan has a cationic property, and the electrospinning positions cannot be easily formed due to the presence of amine groups in C_2, which can be ionized in neutral and acidic pH values. Accordingly, various solutions of chitosan, combined with other polymers such as collagen, polyvinyl alcohol, and polyethylene oxide, have been utilized to facilitate the electrospinning

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procedure. These compounds managed to overcome the agglomeration defect due to the insufficient pulling of strings during the bending of the polymer solution jets, thereby improving the mechanical, thermal, and structural properties as well [16, 17, and 18].

Conventional dressings are mostly cotton-based with some weaknesses, including drying the wound, sticking to the wound and inducing sensitivity in some cases, which can result in a prolonged wound-healing process, painful dressing change, and many other complications. Given the novel developments in fiber production over the recent years, the special properties of dressing materials are expected to improve the wound-healing process. Furthermore, the use of antibiotics as an antibacterial agent has attracted further attention considering the risk of metal nanoparticles [19 and 20]. Ciprofloxacin hydrochloride is one of the effective antibiotics used in the treatment of infected wounds. According to previous studies, the production of drug-containing nanofiber web can lead to gradual drug release and more control on the process of the infectious waste removal [21, 22, and 23]. In many studies, bacterial cellulose (BC) has been introduced as a cost-effective and biological source with diverse applications. Bacterial cellulose is a microbial polysaccharide that is chemically similar to plant cellulose; it is, however, still a topic of ongoing study [24, 25, 26, and 27]. In the present study, given the importance of antibacterial dressings and the porous structure of cellulose, which makes it ideal for absorption and other desired properties, alpha-cellulose nanofibers were extracted from wheat bran as an inexpensive, local, and available source. In this study, drug-impregnated nanofibers were used for the first time to improve the effectiveness and control of the wound-healing process. The FTIR spectra were taken from alpha-cellulose nanofibers after they were impregnated with the antibiotic to confirm the presence of ciprofloxacin. In addition, the effect of the nanofiber ciprofloxacin hydrochloride-impregnated disks on *S. aureus* underwent in-vitro evaluation by the disk diffusion method.

EXPERIMENTAL

Materials

In this study, the alpha-cellulose nanofiber layer based on spring wheat bran was prepared from the Fars Province, Iran. Double-distilled water, hydrochloric acid (98%), trifluoroacetic acid (TFA), methylene chloride (MC).The antibiotic ciprofloxacin was purchased from Sigma Aldrich. The standard strains of *S. aureus* ATCC-25933 were purchased from the Industrial and Mining Research Center of Iran. The Mueller Hinton agar medium was also provided by Merck (Germany).

Impregnation of α-cellulose nanofibers with ciprofloxacin hydrochloride

After preparing the raw material (wheat bran) randomly from Fars Province (Iran), the specimens were first washed with distilled water, dried in an oven, and turned into a fine powder by grinding.

The chemical-mechanical method (Soxhlet-ultrasonic waves) was used to purify the cellulose and to prepare the alpha-cellulose from wheat bran powder. In the chemical method (Soxhlet), lipid and wax were separated from the primary sample (the wheat bran) using a toluene-ethanol solution (1+2). Then, the lipid removal in 300 g of powdered wheat bran was performed to remove lignin using a 7% sodium chlorite solution whose acidity was adjusted to 4-4.5 with acetic acid, and then immersed for 1 hour at 50-60ºC. This step was repeated twice to change the color of the colloidal solution from shade yellow to creamy.

In order to remove hemicellulose and to achieve alpha-cellulose, lignin-free wheat bran powder was immersed in alkaline environment made by 10%NaOH on a magnetic stirrer at 70°C for 1 h. After suspension for 40 minutes, the colloidal solution was poured into three beakers containing 8% NaOH equally, and then sonicated with the ultrasonic apparatus at room temperature for 10 min, 30 min and 60 min.

After studying the results of powdered alpha-cellulose sonicated with FTIR at three mentioned temperatures, the alpha-cellulose powder obtained from 60 min exposure to ultrasonic waves was selected. Then 0.8 g of alpha-cellulose powder was added to an autoclave, labeled A, which could be sealed and protected against liquid evaporation. Then, it was completely dissolved at ambient temperature in a solvent (12% w/v in TFA/MC solution) on a magnetic stirrer with an ice bath for three hours. Thus, color changing in the sample was prevented. The obtained solution was placed in the electrospinning solution tank to prepare the nanofiber layers. The electrospinning operation was carried out at a voltage of 22 kV and a flow rate of 3.5 ml/h, in which the needle–collector distance was 40 mm; for one hour at the end of the process, the nanofibers were collected from the aluminum foil-collector plate. The optimum nanofiber layer was obtained from alpha cellulose. Then, the samples were collected from the obtained nanofiber layer to perform antibacterial tests and heal the superficial wounds of rats. Disks similar to standard antibiogram disks were cut with diameters of 6 mm

and an area of 1 × 1 cm^2 by using sterile scissors under a sterile condition. These layers (nanofibers) were then placed on gauze prepared from the pharmacy, which had been cut down to the size of cellulose fibers. Afterwards, the drug was prepared. Owing to the porous structure of the nanofibers, these sheets seemed to easily absorb the antibiotics from the solution. For drug loading, a ciprofloxacin hydrochloride-containing solution (with a concentration of 3.3% in 0.01 N) was prepared by adding 0.5 g of ciprofloxacin powder (Sigma Aldrich) to 15 ml of hydrochloric acid. Afterward, the alpha cellulose nanofiber- and paper- disks were immersed inside the ciprofloxacin hydrochloride solution at a controlled temperature (25°C). After 24 hours, the disks were removed from the solution and dried at room temperature (Fig.1).

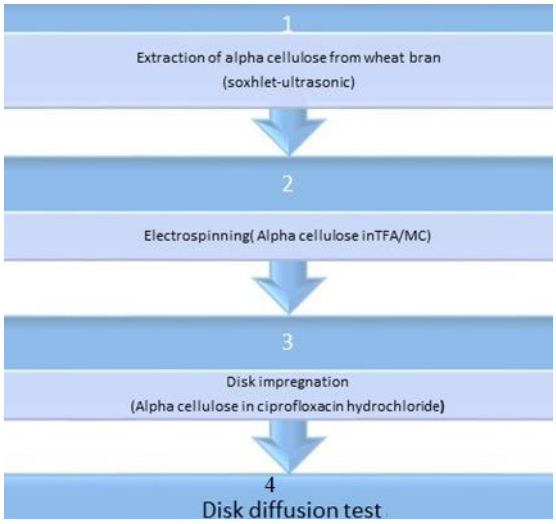


Fig. 1. Test steps

Disk diffusion test

The disk diffusion test was a validated antibiogram method that was performed to investigate the antibacterial effect of the alpha-cellulose nanofibers. The disk diffusion test was carried out by culturing a swab infected with bacterial suspension (equivalent to 0.5 McFarland standards) on the plates containing the Muller Hinton Agar (MHA) medium.

The drug-containing cellulose and paper disks along with standard disks (Iran Daru Co., containing 5 µg of ciprofloxacin per disk) were placed on an MHA medium in a series of three plates with a regular distance by sterile forceps near the flame. In the end, the plates were incubated at 37°C for 24 hours to measure the inhibition zone. To investigate the repeatability of releasing antibiotics from the alpha-cellulose nanofiber layer (examining the hypothesis of whether the alpha-cellulose nanofibers will release the all of the absorbed antibiotics within the first 24 hours or whether it will release it gradually over the course of several consecutive days), the inhibition zone diameter of all disks was determined and recorded after the first 24 hours. Then a fresh suspension of *S. aureus* ATCC-2593 (equivalent to 0.5 McFarland Standard) was prepared. The same suspension was cultured in the plates containing the MHA medium. Finally, all the disks of the first 24 hours were sub-cultured in new plates to study the releasing trend during the second 24 hours. The plates were incubated at 37°C, the results were measured after 24 hours, and their means were recorded for each series. This process was exactly repeated until the inhibition zone diameter reached zero for all the disks.

Determination of the concentration of absorbed ciprofloxacin hydrochloride in alpha-cellulose nanofibers

At first, a solution (a serial dilution) containing 0.625, 1.25, 2.5, and 5 µg/ml of ciprofloxacin hydrochloride was prepared to determine its concentration absorbed in alpha-cellulose and a few layers of alpha-cellulose nanofibers were immersed inside each solution; after 24 hours, they were removed from the solutions and dried. Then, three series (A, B, and C) of plates containing the Muller Hinton Agar medium were prepared and a fresh suspension of *S. aureus* ATCC-2593 (equivalent to 0.5 McFarland Standard) was cultured in each of them. After that, the cellulosic nanofiber layers with a standard disk was placed on to each plate, and the results were recorded by measuring the inhibition zone after 24 hours of incubation at 25°C.

RESULTS AND DISCUSSION

FTIR spectroscopy

The FTIR device (Hartmann & Braun MB-100, Canada) was recruited to study the samples extracted from the wheat bran obtained through mechanical and mechanical (Soxhlet - ultrasonic waves) procedure. All of the obtained graphs were analyzed by ImageJ software. The lignin is bonded to hydroxyl groups of polysaccharides in the cellulose and hemicellulose through etheric linkages. The double bonds in lignin cause darkening of the fiber color. If the lignin is oxidized and its double bonds are broken, the fibers will be whiter. Studies have shown that 20% of lignin consists of hemilignin, which has a relatively low molecular weight and dissolves easily, and its quantity is decreased when processing with alkaline or dilute acid solutions[28-29]. Comparing the spectra of (Fig 2), it can be claimed that the total area of the functional groups in Spectrum 3 is greater than that of Spectrum 2; in fact,

further functional groups are available. As a result, it can be said that the complementary method of ultrasonic waves has been effective in extracting the alpha-cellulose. Perhaps because the ultrasonic method, in contrast to the Soxhlet, causes better mixture of bran and solvent extractor, and thus extracts more and better the alpha-cellulose. The cellulose molecules are linear completely, and have a strong affinity to form intermolecular hydrogen bonds. Hence, groups of cellulose molecules are combined with each other to make microfibers. By studying the FTIR results of the alpha-cellulose extracted from the wheat bran at 10min, 30min and 60min ultrasonication, the alpha-cellulose samples extracted from 60 min exposure to ultrasonic waves were selected for further studies (Fig.2).

The FTIR spectrum of the ciprofloxacin-containing alpha cellulose nanofibers were tested to confirm the presence of ciprofloxacin hydrochloride in the nanofibers. Figs. 3a and 3b show the FT-IR spectra of the ciprofloxacin-containing nanocellulose disk prepared from wheat bran-extracted alpha-cellulose. The band in 3099 cm^{-1} is related to the aromatic ring-hydrogen bond (Ar-H) in the drug structure and the band in 3531 cm^{-1} is associated to the hydroxyl (OH) group of the drug as well as the cellulose structure. The presence of other groups of the drug structure were confirmed by the following bands in the FT-IR results: the bands in 1,707 and 1622 cm^{-1} confirm existence of carbonyl (C=O) and nitrogen–hydrogen (NH) functional groups in the drug structure, respectively. The band observed in 1456 cm^{-1} is representative of the carbon–hydrogen (C-H) bond in the cellulose structure as well as the carbon–carbon (C-C) bond in the aromatic rings of ciprofloxacin; the band in 1149-1319 cm^{-1} can be associated to the carbon–fluorine (C-F) bond of the drug structure as well as the carbon–oxygen (C-O) bond in the cellulose structure; the band in 838–708 cm^{-1} indicates the carbon–hydrogen bond in both the alpha-cellulose and the ciprofloxacin structure; finally, the band in 935–986 cm^{-1} can be related to the hydroxyl (OH) group of the carboxylic acid group.

The comparison of the two spectra (Figs. 3b and 3c) showed multiple differences in the type and the location of the peaks. Fig. 3b shows that ciprofloxacin hydrochloride was well positioned on alpha cellulose to form a new compound (Fig. 3).

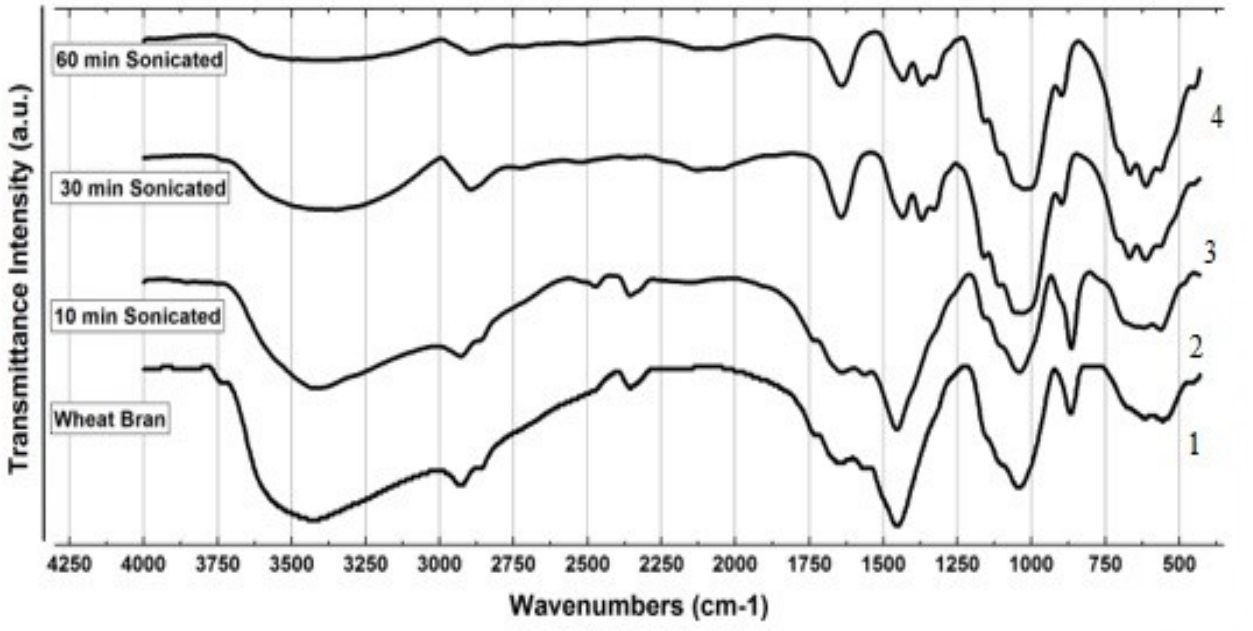
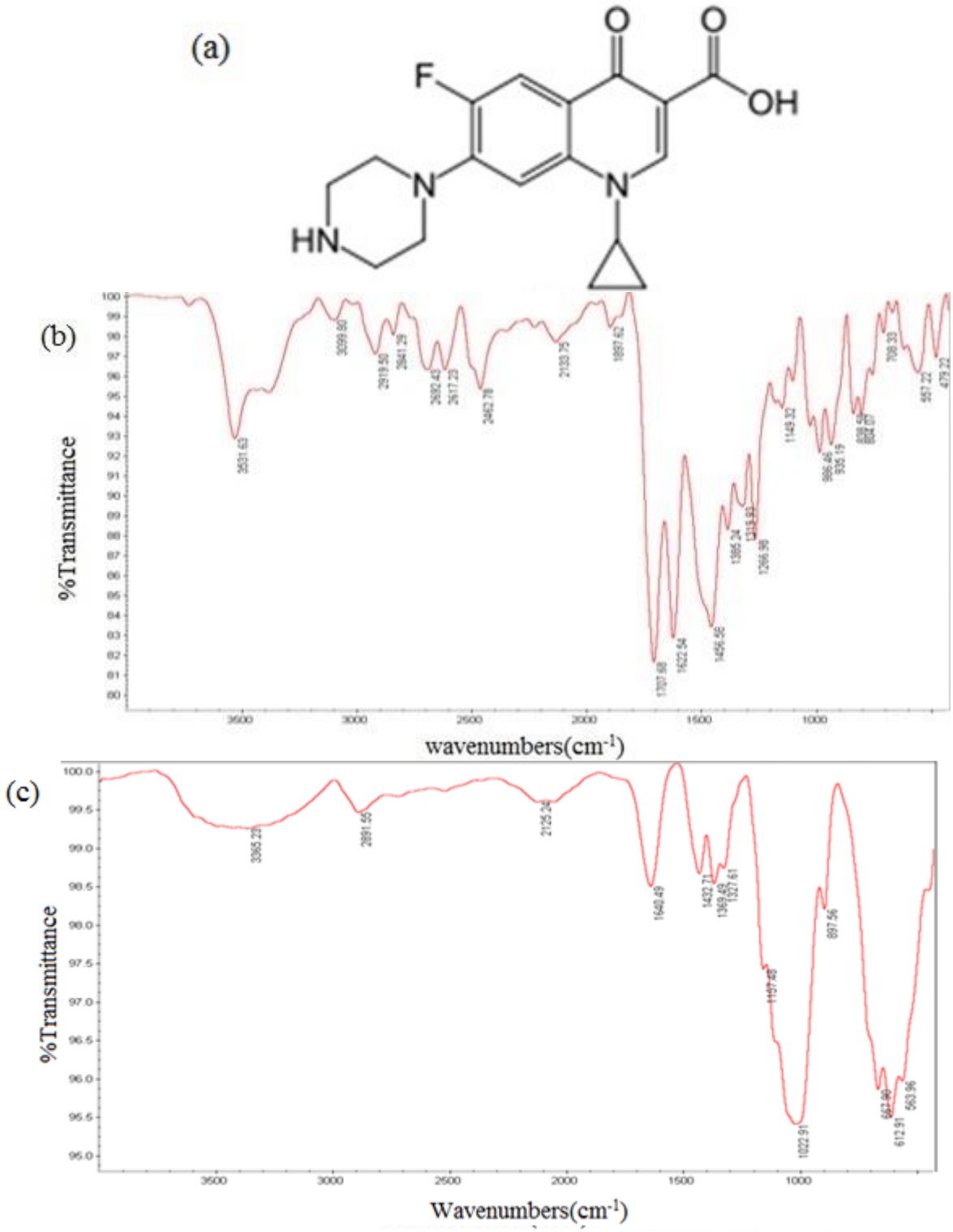


Fig. 2. Comparison of FTIR spectra of the wheat bran, ultrasonic for 10 min, 30 min and 60 min



Figs. 3(a). Structure of ciprofloxacin hydrochloride, 3(b): Ciprofloxacin-containing nanocellulose disk prepared from wheat bran-extracted alpha-cellulose, 3(c): Wheat bran-extracted Alpha-cellulose nanofibers

SEM

The surface microstructure was examined by SEM. First, the specimens were coated with gold in a thickness of 2 nm. The imaging was then taken using a TESCAN VEGALL SEM .Cellulose nanofibers were observed and investigated within a diameter range of 200–1,000 nm at a scale of 10,000 units with a voltage ranging from 5 to 10 kV. Images of electrospun wheat bran-extracted alpha-cellulose nanofibers are presented in Figs. 4a and 4b

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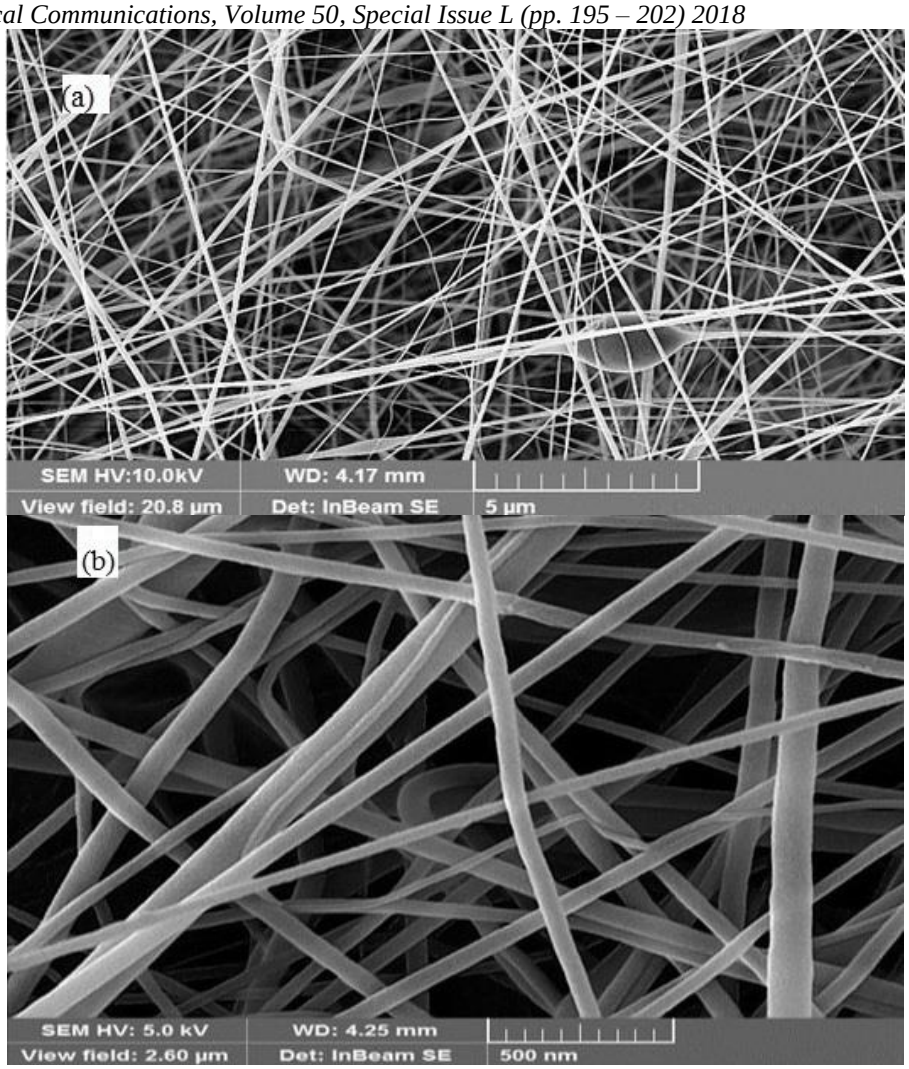

Fig. 4. SEM images of alpha-cellulose with 12 w/v% in TFA/MC

Antibiogram studies

The mean diameter and thickness of the alpha-cellulose nanofibers embedded on the gauze were 6 mm and 0.3 mm, respectively. In addition, the mean diameter and the thickness of the standard paper disks were 6.4 mm and 1 mm, respectively. Accordingly, the size of these two types of disks were different, which can be seen in Fig. 5.

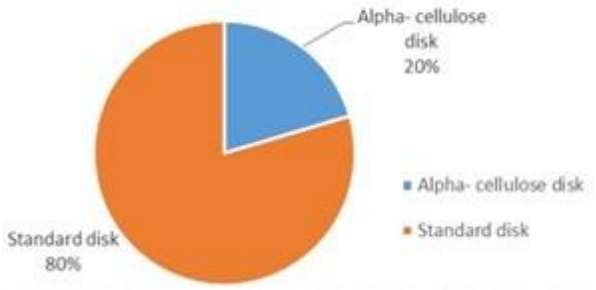

Fig. 5. Difference in the area and the volume of alpha-cellulose nanofibers and standard paper disks.

According to Fig. 5, the ratio of the cellulose disk volume to the paper disk volume was 25.72%. This difference indicates that if these two disks are immersed in the same antibiotic solution, the paper disk will absorb more antibiotics due to its larger volume by assuming the same solute affinity for binding itself to both disks. This issue can be verified after measuring and comparing the inhibition zones of the three disks (Figs. 6a,b).

As *S. aureus* is one of the most prevalent pathogens of burn infections and skin lesions, previous studies have shown the ability of silver cation-containing microbial cellulose disks to create the inhibition zone on *S. aureus* and E. coli media [30]. The present study is aimed at comparing the effect of drug-containing alpha-cellulose nanofiber disks, ciprofloxacin hydrochloride-containing papers, and the standard disk of this antibiotic on *S. aureus*. A comparison of the data in Table 1 by the Kruskal–Wallis test on the basis of the nonparametric method indicated no significant difference between cellulose and the paper disks ($P < 0.05$).

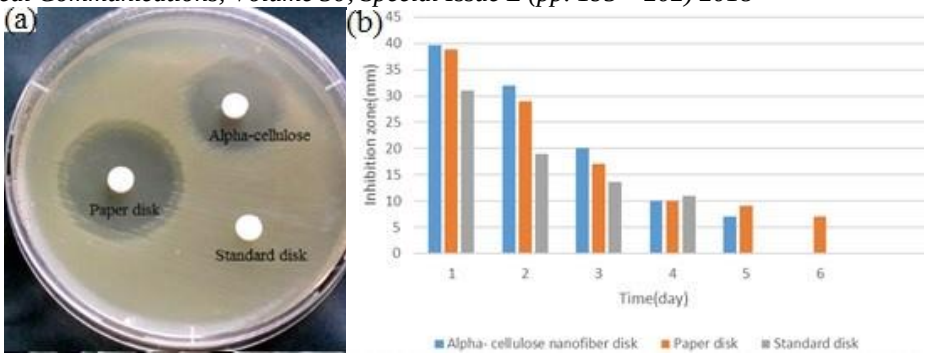

Fig. 6(a). Inhibition zone diameter (mm) during the fifth period of 24 hours, 6(b): Inhibition zone diameter (mm) of different disks after six days

The control paper disk showed an inhibition zone in the *S. aureus* culture media. On the other hand, considering that the cellulose disk volume was about 25% of the paper disk and that both disks were immersed in the same antibiotic solution, it can be said that the cellulose disk has a high absorption capacity as it absorbs the same concentration of ciprofloxacin hydrochloride by almost a quarter of the volume of the paper disk. The reason for this claim can be attributed to the large and regular pores in the cellulose tissue, which is the major potent of cellulose in absorbing and releasing this antibiotic. As shown in Fig. 6b, there is a large difference between the inhibition zone from the standard and the paper disks. These two disks are made of paper with an equal volume. Therefore, this difference can arise due to a difference in the antibiotic concentration. On the other hand, Table 1 shows that the power of drug-containing cellulose and paper disks is due to the repeatability of releasing. Moreover, it confirms a lower concentration of ciprofloxacin hydrochloride of the standard disk since the release repeatability of this disk is lower than the paper disk. A comparison of the inhibitory zone of control and cellulose paper disks for several consecutive days indicated that the cellulose disk was weaker than the paper disk in terms of release repeatability. This difference can be justified due to the greater volume ratio of the paper disk to the cellulose disk (25.72%). Based on the aforementioned information, it can be concluded that ciprofloxacin hydrochloride-impregnated alpha-cellulose has suitable in-vitro power in preventing the growth and the proliferation of *S. aureus*.

According to Table 2, the ciprofloxacin concentration in cellulose disks can be calculated according to the antibiotic concentration in a standard disk (5 μg) with a mean inhibition zone of 31.3 mm using simple proportionality. Thus, the amount of ciprofloxacin in the cellulose disks containing 0.625% antibiotic solution with the mean inhibition zone of 31.6 mm will be about 5.04 μg. Similarly, the antibiotic concentration can be determined in the rest of the disks as shown in Table 2.

Table 1. Inhibition zone diameter (mm) of different pads after six days

Days	Alpha-cellulose nanofiber disk containing drug(mm)	Paper disk containing drug(mm)	Standard disk(mm)
1	39.6	38.9	31
2	32	29	19
3	20	17	13.6
4	10	10	11
5	7	9	0
6	0	7	0
7	0	0	0

Table 2. Comparison of the inhibition zone diameters obtained from cellulose disks containing different ciprofloxacin concentrations and the standard disk in the three series of plates A, B, and C

Types of disk	Inhibition zone A B C	Mean	Standard deviation	Cip(µg)
Standard disk	30 33 31	31.3	1.44	5
Alpha-cellulose nanofiber disk containing 5% drug	40 40 41	40.33	0.22	6.44
Alpha-cellulose nanofiber disk containing 2.5% drug	37 34 39	36.6	4.2	5.84
Alpha-cellulose nanofiber disk containing 1.25%drug	31 33 35	33	2.6	5.27
Alpha-cellulose nanofiber disk containing 0.625% drug	30 31 34	31.6	2.89	5.04

CONCLUSION

The results obtained in this study revealed that the ultrasonic waves improved the stages of alpha-cellulose extraction from wheat bran at the study times. In fact, the cavitation bubbles produced near the cellulose surface caused the cellulose tissue swelling to absorb the solvent and release the compounds from the tissue to the solvent. Its contents were released in the environment. The FTIR images showed that the successful extraction processing under the influence of ultrasonic waves to access the alpha-cellulose and the optimal conditions were detected after 60 minutes of exposure to waves. In this study, anew combining method was presented for Antimicrobial dressings regarding the findings of this research appropriate morphology of the nanofibers prepared from extracted α-cellulose from wheat bran was obtained with the TFA/MC mixed solvent. the alpha-cellulose nanofibers synthesized by electrospinning method and impregnated with antibiotic ciprofloxacin hydrochloride compared to the standard disk have a greater inhibitory effect on *S. aureus* ATCC-25933.

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