Original research

Antimicrobial activity of nanocellulose conjugated with melanin

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http://jabs.eu5.org/

Received: July. 5, 2015
Accepted: July. 28, 2015

Vol. 1, No. 3, 2015, pages 229-242.

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Abstract

In this study, nanocellulose was synthesized by acid hydrolysis, modified by citric acid, conjugated with amine melanin by carbodiimide cross-linker. Then, its antimicrobial property was evaluated by microdilution method, and compared with melanin and nanocellulose. The results showed that nanocellulose had little antimicrobial activities, but high antifungal and antibacterial effect against standard strains of *Candida albicans, Aspergillus niger, Staphylococcus aureus,* and *Escherichia coli* was seen for melanin-conjugated nanocellulose, same as the effect with melanin alone. The authors suggest that melanin-conjugated nanocellulose must be evaluated further to use in industry as an antimicrobial structure in food packaging, inside foodstuff, and textile materials.

Key words:

Antimicrobial activity; Cellulose nanoparticles; Lysozyme

Introduction

Nanocellulose has high surface/volume ratio, crystallity, and dispersion ability. Interestingly, its good stability against proteolytic enzymes, acids, temperatures, and high biodegradability are very important properties [1]. According to these properties, different applications have been suggested for nanocellulose, e.g., as a reinforcing filler in nanocomposites, as a strengthening element in paper, as an adsorbent, as a carrier of genes and drugs in medicine, and as a degradable film in packaging [2]. Technically, nanocellulose can be synthesized by different methods, but acid hydrolysis has been used in the most studies. After hydrolysis, disintegration process is done by high-pressure homogenizer, ultrasound device, and ball miller [3-4]. In aspect of chemistry, nanocellulose has many active hydroxyl groups and can be modified by different molecules. Modified nanocellulose maybe has different properties, and can be applied in specific conditions [5]. Antimicrobial activity is an important property which can be achieved by modification or conjugation of nanocellulose with different antimicrobial agents such as metal nanoparticles, metal oxide nanoparticles, organic compounds, etc [6]. One of natural antimicrobial agents is melanin (diallyl thiosulfinate), which inhibits wide range of Gram-negative and Gram-positive bacteria, fungi, parasites, and viruses [7]. In the garlic clove cells, alliin is converted to pyruvate, ammonia, and melanin by the pyridoxal 5′-phosphate (PLP)-dependent alliinase, and then melanin react with thiol groups of various enzymes, such as thioredoxin reductase, alcohol dehydrogenase, RNA and DNA polymerase, and cysteine proteinase [8]. Moreover, melanin has anti-oxidant, anti-cancer, anti-inflammatory, anti-thrombotic, anti-atherosclerotic, anti-serum lipid, and pro-circulatory effects [9-10]. Because melanin has no active functional groups, the attachment of melanin to other chemical molecules is hard, and must be modified before conjugation. Amine-melanin is one modified molecule which can conjugate with other molecules by its amine group. On the other hand, alliin has carboxyl and amine group, and can be used for conjugation, but its antimicrobial

activity has been not reported [10-11]. In literature, there is no study on conjugation of nanocellulose by melanin or alliin. Thus, the aim of this study was to synthesize melanin-conjugated nanocellulose, and then its antifungal and antibacterial properties were investigated by microdilution method.

Materials and methods

Materials

To synthesis nanocellulose, the batting (crude cellulose) which manufactured by My Baby Company was used. $RPMI_{1640}$ was purchased from Invitrogen, UK. Melanin and N-ethyl-N-(dimethylaminopropyl) carbodiimide (EDC) were provided from Sigma-Aldrich Chemical Company (St Louis, MO). Other chemicals including nitric acid, sulfuric acid, formaldeide, sodium hydroxide, amine-melanin, and dimethylsulfoxide (DMSO) were sourced from Zyst Fannavar Shargh Company, Yazd, Iran.

Nanocellulose synthesis

To synthesis nanocellulose, acid hydrolysis method was done according to Loelovich et al. study with some modifications (3) [3]. Before synthesis of nanocellulose, 5g cellulose was treated with 25 mL of 5M NaOH at 37 ºC for one hour, and then rinsed with distilled water (DW). In the next step, 25 mL of 1M DMSO was added to washed cellulose, and incubated at 37 ºC for one hour, too. Finally, DMSO treated cellulose was washed three times with DW, and used for synthesis of nanocellulose. In the next step, serial concentrations (90%, 80%, 70%, 60% and 50%) of acid mixture were prepared. It must be mentioned that the initial acid mixture had sulfuric acid (85%), nitric acid (5%) and water (10%). Then, 1g washed cellulose was separately added to 1 mL of serial concentrations of acid mixture, and incubated at room temperature for 30 minutes. The completely

hydrolyzed cellulose with milky color was chosen, and 2 mL of 5M NaOH was gently added to it. In the final step, tubes were centrifuged at 3000 rpm for 5 minutes, and then nanocellulose pellets were washed by DW three times. Nanocellulose was suspended in DW, shacked 5 minutes, and stored in 5 ºC.

Preparation of conjugated nanocellulose

Briefly, 500 mg of nanocellulose was added to 5 mL of 7% citric acid and 5% sodium hypophosphite monohydrate, and shacked for 30 minutes at room temperature. Then, modified nanocellulose was centrifuged for 5 minutes at 5000 rpm, and washed with DW. Then, 1 mL of EDC (233 mg/mL) and 1 mL of amine-melanin (100 mg/mL) were added to 500 mg of modified nanocellulose, incubated at 37 ºC for 1 hour, centrifuged at 5000 rpm, and washed with DW. The schematic of this reaction is shown in Figure 1. In the final step, serial concentrations (1000, 500, 250, 125, 62.5 µg/mL) of nanocellulose, melanin-conjugated nanocellulose, and melanin alone were prepared in $RPMI_{1640}$.

Characterization of nanocellulose

The structure, size distribution, and surface composition of both nanocellulose and melanin-conjugated nanocellulose were studied by scanning electron microscopy (SEM) (Hitachi, S-2400), dynamic light scattering (DLS) (Malvern Instruments, Italy), and Fourier transform infrared spectroscopy (FTIR) (ELICO, India), respectively. For SEM investigation, all samples were coated with gold by sputtering apparatus and then studied at 15 Kv. The FTIR was used to confirm conjugation. In this experiment, the spectrums of melanin, nanocellulose, and melanin-conjugated nanocellulose were investigated at 400-4000 cm^{-1}.

The evaluation of antimicrobial property

According to NCCLS, broth microdilution method was used for evaluation of antimicrobial susceptibility of melanin, nanocellulose, and melanin-conjugated nanocellulose. Four standard microbial strains including *Candida albicans* ATCC 29458 *(C.albicans), Aspergillus niger (A.niger), Staphylococcus aureus (S.aureus),* and *Escherichia coli (E.coli)* (---) were used in this study which were obtained from Iranian Research Organization for Science and Technology. Firstly, fungal and bacterial strains were inoculated onto Sabouraud dextrose agar and nutrient agar, respectively. Fungal and bacterial plates were incubated for 48 hours at 25 ºC and 37 ºC, respectively. Then, one colony of each strain was separately added to 5 mL of $RPMI_{1640}$-2% glucose medium. The final density was 2×10^4 cells/mL and its optical density (OD) was 0.1 at 260 nm. In the next step, 100 µL of melanin, nanocellulose, and melanin-conjugated nanocellulose at serial concentrations was separately incubated with 100 µL of microbial suspension. Then, fungal and bacterial strains were incubated at 25 ºC and 35 ºC for 48 hours, respectively.

In this experiment, negative and positive controls were included. Microbial suspensions not treated with melanin, nanocellulose, or conjugated nanocellulose were considered as negative control. For positive control, fungal strains were exposed to 2 µg/mL nystatin, and bacterial strains were treated with 1 µg/mL ceftriaxone. After incubation, the OD of each well was read at 405 nm by ELISA reader (Novin Gostar, Iran), and minimum inhibitory concentration (MIC) of melanin, nanocellulose, and melanin-conjugated nanocellulose against different strains was calculated. In this study, MIC_{50} and MIC_{90} were measured, according to negative control.

Statistical analysis

The results are shown as the mean ± standard deviation (SD) with three independent tests. Parametric test (Student's *t-test*) was applied to evaluate the significant difference by use of SPSS software (V.16.0 for Windows; SPSS Inc., USA). $P<0.05$ was considered as significant difference.

Results

Characterization of nanoparticles

SEM images of nanocellulose and melanin-conjugated nanocellulose are shown in Figure 2a and Figure 2b, respectively. According to these figures, both nanocellulose and melanin-conjugated nanocellulose are approximately spherical. Figure 2c and Figure 2d show the distribution size of nanocellulose and melanin-conjugated nanocellulose. As shown, the distribution size of nanocellulose and melanin-conjugated nanocellulose is about 100-150 nm and 100-170 nm, respectively. Also, FTIR spectrum of nanocellulose (a), citric acid modified nanocellulose (b), amine melanin (c), and melanin-conjugated nanocellulose (d) is observed in Figure 3. Generally, spectrum of melanin-conjugated nanocellulose confirmed attachment of amine melanin to modified nanocellulose, i.e., both specific spectrum of melanin and modified nanocellulose were seen in spectrum of melanin-conjugated nanocellulose.

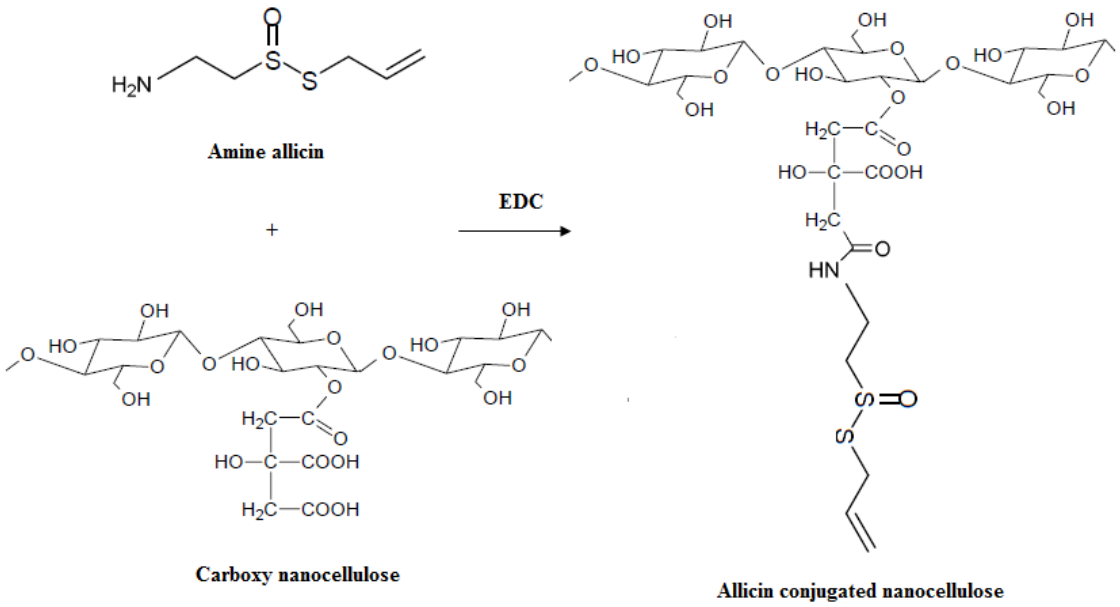

Figure 1. The schematic of conjugation between carboxy nanocellulose and amine melanin by EDC crosslinker.

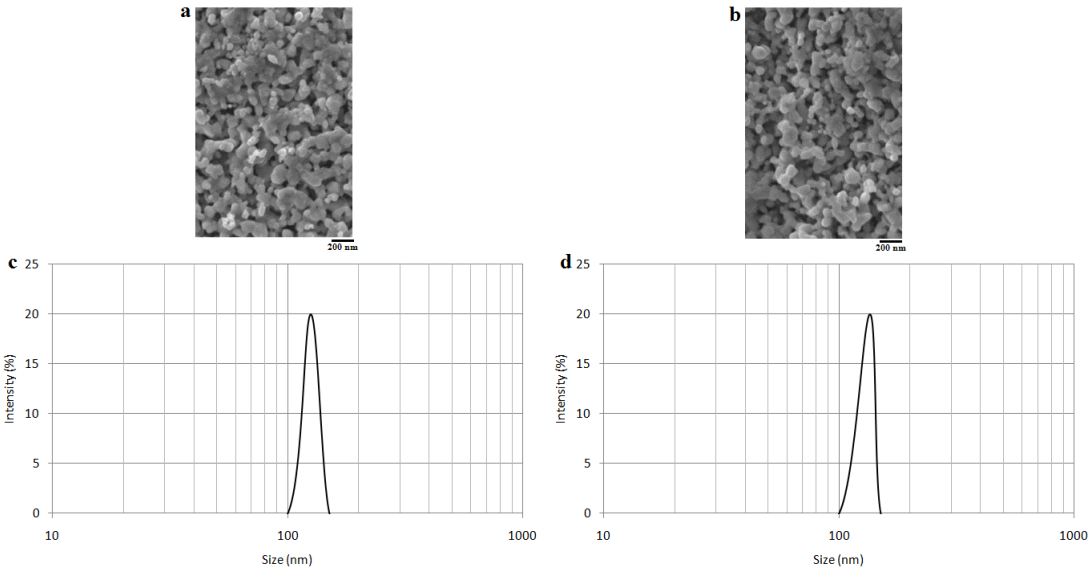

Figure 2. The SEM images of nanocellulose (a) and melanin-conjugated nanocellulose nanoparticles (b). The DLS graph of nanocellulose (c) and melanin-conjugated nanocellulose (d).

Vol. 1, No. 3, 2014, pages 229-242.

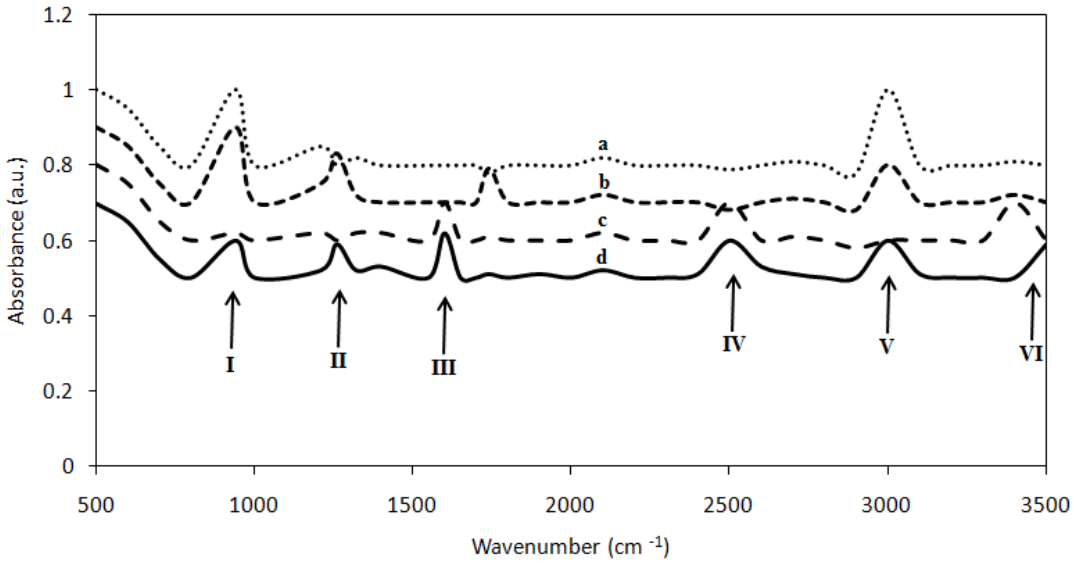

Figure 3. The FTIR spectrum of nanocellulose (a), citric acid modified nanocellulose (b), amine melanin (c), and melanin-conjugated nanocellulose. I (940 cm^{-1}), II (1260 cm^{-1}), III (1600 cm^{-1}), IV (2500 cm^{-1}), V (3000 cm^{-1}), and VI (3500 cm^{-1}) are vibration peaks.

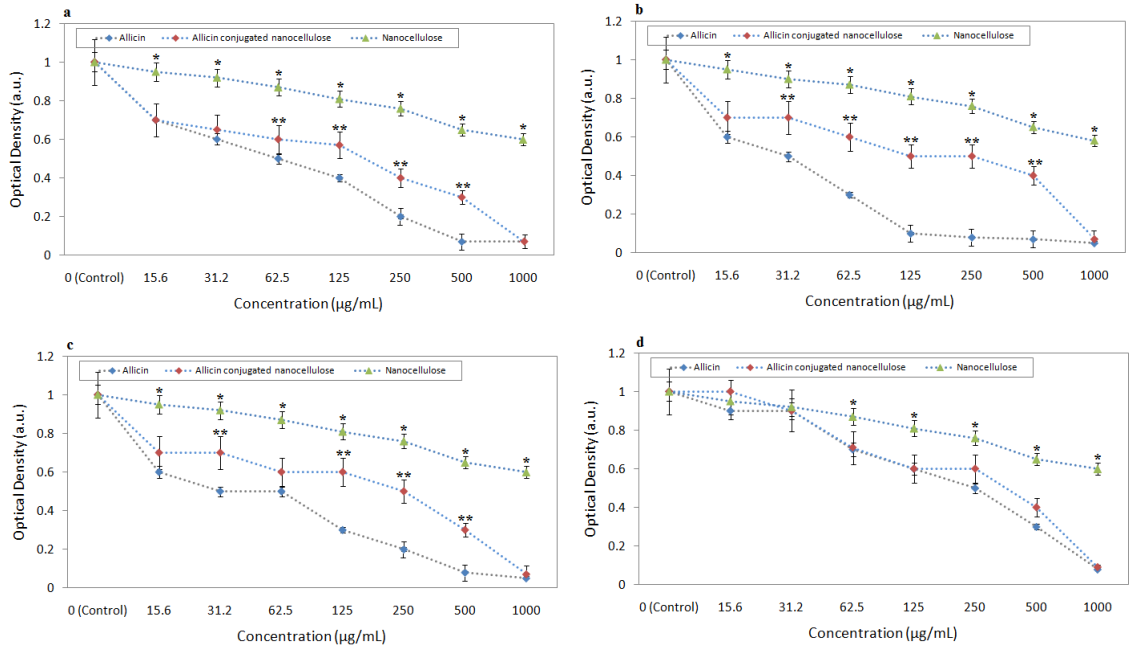

Figure 4. The dose-response graph. Different concentrations of nanocellulose, melanin-conjugated nanocellulose, and melanin were separately added with *E.coli* (a), *S.aureus* (b), *A.niger* (c), and

Vol. 1, No. 3, 2014, pages 229-242.

C.albicans (d) suspension, and incubated 48 hours at 25 ºC and 35 ºC for fungal and bacterial strains, respectively. The OD of each well was read at 405 nm by ELISA reader. All data are shown as mean ± SD with n=3. *P<0.05 compared with melanin and melanin-conjugated nanocellulose at the same concentration. **P<0.05 compared with melanin at the same concentration.

MIC results

The MIC_{50} and MIC_{90} of nanocellulose, melanin-conjugated nanocellulose, and melanin against two fungal and two bacterial strains are shown in Table 1. As shown, the MIC_{50} and MIC_{90} of melanin-conjugated nanocellulose against all strains were 500 and 1000 µg/mL, respectively. As shown, melanin had the least MIC_{50} and MIC_{90} compared with nanocellulose and melanin-conjugated nanocellulose against all strains. Also, Figure 4a, Figure 4b, Figure 4c, and Figure 4d demonstrate the effect of serial concentrations of all these materials against *E.coli*, *S.aureus*, *A.niger*, and *C.albicans*, respectively. As demonstrated, nanocellulose had few antifungal and antibacterial properties, and the highest effect was seen at concentration of 1000 µg/mL. Both melanin-conjugated nanocellulose and melanin had antifungal properties against *C.albican* and *A.niger*, and antibacterial properties against *S.aureus* and *E.coli*. In case of all strains, the MIC_{50} and MIC_{90} of melanin was less than melanin-conjugated nanocellulose. There were significant differences between MIC (both 50 and 90) of melanin-conjugated nanocellulose nanoparticles vs. melanin (P<0.05) for all strains. Also, the inverse relationship was observed between OD and concentration against melanin-conjugated nanocellulose and melanin in all strains.

Table 1. The MIC_{50} and MIC_{90} of nanocellulose, melanin-conjugated nanocellulose, and melanin against fungal and bacterial isolates.

		Isolates			
		C.albicans	*A.niger*	*S.areus*	*E.coli*
MIC_{50} (µg/mL)	Nanocellulose	>1000*	>1000*	>1000*	>1000*

	Melanin	250**	125**	62.5**	125**
	Melanin conjugated nanocellulose	500	500	500	500
MIC_{90} (µg/mL)	Nanocellulose	>1000¥	>1000¥	>1000¥	>1000¥
	Melanin	1000	500¥¥	250¥¥	500¥¥
	Melanin conjugated nanocellulose	1000	1000	1000	1000

*<0.05 compared with MIC_{50} of melanin and melanin conjugated nanocellulose on the same isolate

**<0.05 compared with MIC_{50} of melanin conjugated nanocellulose on the same isolate

¥<0.05 compared with MIC_{90} of melanin on the same isolate

¥¥<0.05 compared with MIC_{90} of melanin conjugated nanocellulose on the same isolate

Discussion

In the present study, nanocellulose were synthesized by hydrolysis method, modified by citric acid, and then conjugated with amine melanin. Then, antibacterial and antifungal properties of nanocellulose, melanin-conjugated nanocellulose, and melanin were studied by microdilution method.

As noted, acid hydrolysis which has been introduced in other studies was used to synthesize nanocellulose, because this method is easy and inexpensive [2, 4]. For this purpose, crude cellulose was firstly washed with NaOH and DMSO, which our aim was to eliminate cellulose impurity. The authors propose that different impurities may affect synthesis of nanocellulose, such as other chemical reactions. In the second step, washed cellulose was incubated with serial concentrations of acid mixture (90%, 80%, 70%, 60% and 50%), because synthesis of nanocellulose depends mainly on acid concentration. We found that acid mixtures at concentration of 80% and 90% were not suitable to synthesize nanocellulose, and they led to complete reduction of cellulose. Moreover, the concentration of 60% and 50% had no enough power to synthesize nanocellulose, and led to partial hydrolysis. We observed that concentration of 70% was exactly suitable, and produced nanocellulose at room temperature with desired size. In previous studies, synthesis of nanocellulose

has been reported at different concentrations of sulfuric acid (44-70%), temperatures (25-70 ºC), and hydrolysis times (0.5-24 hours) [2, 4]. In the second step, nanocellulose was modified with citric acid, which the reason of modification was to add carboxyl groups to nanocellulose. As mentioned, melanin has no active functional groups, and the attachment of melanin to other chemical molecules is hard. Amine-melanin is one modified molecule which can conjugate with other molecules by its amine group. EDC, as known crosslinker, conjugates carboxyl and amine, and was used to conjugate modified nanocellulose and amine melanin (Figure 1). As noted in result section, FTIR spectrum was confirmed attachment of these molecules (Figure 3). The microdilution results showed that nanocellulose had same antifungal and antibacterial activity, but these properties were not very high, i.e., the MIC_{50} and MIC_{90} of nanocellulose was >1000 against all strains. We hypothesize nanocellulose cannot target cell wall, cell membrane, and active enzymes of bacterial and fungal strains. But melanin and melanin-conjugated nanocellulose had powerful antibacterial and antifungal properties. In case of *A.niger*, *S.aureus*, and *E.coli*, melanin had higher antibacterial and antifungal activity (with less MIC_{50} and MIC_{90}) than melanin-conjugated nanocellulose. The reason of this pattern is maybe due to the quantity of active molecules in same concentration of melanin and melanin-conjugated nanocellulose. On the other hand, melanin-conjugated nanocellulose might not damage cytoplasmic enzymes same as melanin, because of its large size. The large size of melanin-conjugated nanocellulose might only damage surface enzymes and proteins. In case of *C.albicans* (Figure 4d), same pattern of antimicrobial property was seen for melanin-conjugated nanocellulose and melanin. The authors hypothesize that melanin-conjugated nanocellulose and melanin may have different mechanisms of inhibition on *C.albicans*. Although melanin-conjugated nanocellulose has melanin part, but this structure may affect on bacterial and fungal strains with different routes, as described in melanin. The related mechanisms and its uptake must be investigated in future studies.

There was no study on conjugation of melanin and nanocellulose, and also no report was found on its antimicrobial activity. This study was in consistent with previous studies. Melanin exhibits a powerful antibacterial activity against different Gram negative and Gram positive bacteria such as *Escherichia, Staphylococcus, Streptococcus, Salmonella, Proteus, Klebsiella, Clostridium, Bacillus, and Mycobacterium* [12]. Also, melanin has an antifungal activity against *Aspergillus*, *Cryptococcus*, *Candida*, *Trichophyton*, *Epidermophyton*, and *Microsporum* [13-15]. In aspect of mechanism, the rapid reaction of thiosulfinate and thiol groups of enzymes leads to its inhibition and biocidal activity. This reaction destructs the important microbial enzymes, e.g., thioredoxin reductase, RNA and DNA polymerase, alcohol dehydrogenase, cysteine proteinase, etc.

Regarding its application, the authors suggest that the melanin-conjugated nanocellulose can be used as preservative in food or as an antimicrobial agent in packaging or textile. But it must be mentioned that its stability should be evaluated in future studies. Taken together, nanocellulose can be conjugated with melanin, and has antifungal and antibacterial activity against *C.albicans*, *A.niger*, *S.aureus*, and *E.coli* strains.

Conclusions

This study showed that although cellulose nanoparticles have little antibacterial and antifungal activities, but cellulose nanoparticles conjugated with melanin had high antimicrobial effects against *C.albicans*, *A.niger*, *S.aureus*, and *E.coli*, and may be used in industry as an antimicrobial agent in packagings, in foods, and on textile materials.

Acknowledgments

This paper has been extracted from MSc thesis of Mr Aliasghar Behzadi, and financially supported by Shahid Sadoughi University of Medical Science, Yazd, Iran. The authors thank the laboratory staff of the Yazd Pajoohesh medical lab.

Conflicts of interest

No conflict of interest was addressed.

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