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Investigation of Antimicrobial Effect and Mechanical Properties of Modified Starch Films, Cellulose Nanofibers, and Citrus Essential Oils by Disk Diffusion Method

Omid Daliri Shamsabadi*

Chemical Engineering Department, Faculty of Chemical Engineering, Islamic Azad University, Shahreza Branch, Postal Code 8648146411, Shahreza, Isfahan, Iran

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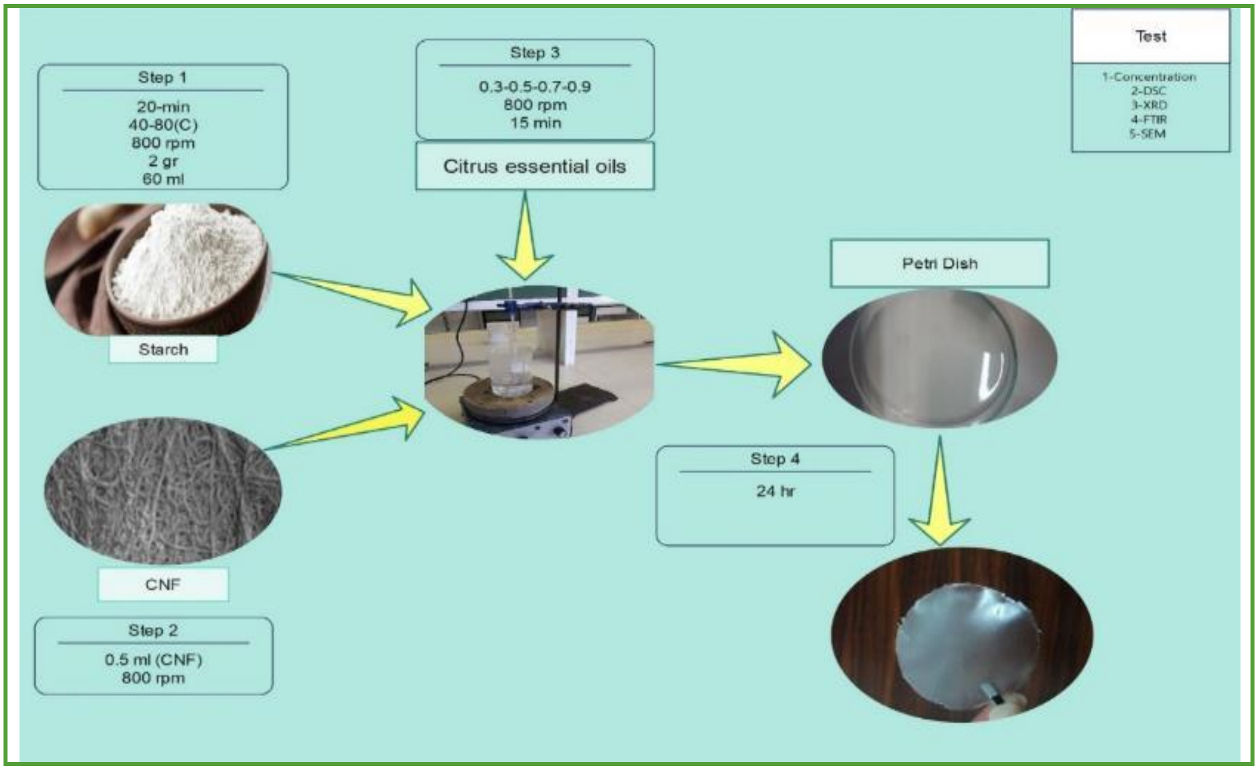
ABSTRACT

This research investigated the mechanical and thermal properties of starch films reinforced with cellulose Nanofibers and lemon essence, which are used in the food packaging industry. One of the aims of this study is to examine the effect of each parameter (alone and together) and evaluate the amount of starch, cellulose Nanofibers, and lemon essence in the manufacture of films. Furthermore, finding a relation between the different amount of used materials that provides the best properties is another research goal. Biodegradable nanocomposite films based on starch and cellulose nanofibers reinforcing agents were prepared via solvent casting. The physical properties of as-prepared starch and cellulose Nano fibers films, such as thickness, colour, and structure, were studied by XRD and scanning electron microscope (SEM). Moreover, DSC and infrared spectroscopy (FT-IR) were used to investigate their thermal properties, such as glass transition temperature (T_g), melting temperature, and crystallization percentage. The disk diffusion method evaluated the antimicrobial effect of biodegradable films containing different concentrations of essence against Escherichia coli and Staphylococcus aureus bacteria. SEM images show uniform and better dispersion of starch and cellulose Nano fibers containing lemon essence, especially in starch and cellulose Nanofibers on the surface (SNE-4).

Corresponding authors, email: omiddaliry@yahoo.com (O. Daliri Shamsabadi).
Tel.: 09137167182.

Graphical Abstract



Introduction

The cell wall in every plant cell is a layered and complex structure. It consists of a thin primary wall that surrounds a secondary wall. The secondary wall consists of three layers; the thick middle layer plays an important role in maintaining the shape and strength of the plant; because it consists of many Lignocellulosic fibers. Lignocellulosic fibers are composites in which polysaccharides and glycoprotein complexes such as lignin and hemicellulose surround tough cellulose microfibers. Cellulose is the most abundant biopolymer that is obtained from agricultural products as a polymer source and can replace petroleum polymers. Unlike synthetic fibers, cellulose fibers have better mechanical properties and lower density. In the last decade, we have seen a lot of interest in using Nano cellulose as a stable and biodegradable alternative for packaging films [1]. In general, three types of cellulose (cellulose nanofibers, cellulose Nanocrystals, and bacterial nano cellulose) have been introduced. Cellulose nanocrystals and cellulose nanofibers have been combined as reinforcing agents in various biopolymers to make green composites [2]. It has been reported that the films produced from nano cellulose can be easily recycled and converted back into a packaging film without greatly reducing its similar properties [3]. The most important reason for this is its carbon neutrality, non-toxic nature, recyclability, and sustainability. Improved properties for the packaging of foods that require less moisture, for example, nuts, dried fruits, and spices have been achieved through multilayer packaging of cellulose nanofibers on polyethylene terephthalate (PET) lamination, and the resulting film was coated with low-density polyethylene through extrusion, creating a three-layer structure [4]. The barrier performance of this multilayer film was better than that of ethylene vinyl alcohol film. With the increase of cellulose nanofibers (from 1 to 7), air and oxygen permeability decreased by approximately 70% and 10^4 , respectively [5]. However, using nano cellulose alone is not desirable, because it provides poor resistance to water vapor, making it unsuitable for use at high humidity levels. The presence of nano cellulose in the film as a filler or coating increases the inhibitory properties, including gases and water vapor, and improves the necessary mechanical properties. At present, a wide range of applications of nano cellulose can be seen in the packaging industry. The original and pure starch is composed of two major macromolecular compounds named amylose and amylopectin. Amylose is a relatively linear polymer consisting of alpha-4 and 1-glucose units, which have excellent film-making capabilities and are used in the preparation of colorless, odorless, non-printing, and strong films. Meanwhile, amylopectin is a highly branched polymer composed of short chains that are connected at the branched positions of alpha-6 and 1 and are repeated every 25 to 30 glucose units [6, 7]. Starch-based films have several advantages, such as high elasticity and good protective properties against oxygen. These films have the same physical characteristics as common and conventional packaging plastics in terms of transparency, smell, and design. In addition, starch-based films are reported to be non-toxic, which helps to increase the acceptance and acceptability of these films as new packaging alternatives to conventional packaging. However, starch is very hydrophilic, starch-based films are very sensitive to water, have high solubility in water and have weak protective properties against water vapor. The mechanical resistance is low, its tensile strength is less than 5 MPA [8]. During the preparation of these films by heat treatment method, there is no need for much water and moisture. High pressure conditions with high shear stress were applied in the presence of plasticizers such as water and glycerol, which caused starch granules to break, increased the speed of water transfer to starch molecules and breaks the amylopectin network and release amylose. Starch has many uses. In fact, starch can be converted into some chemicals such as acetone, ethanol, and organic acids and used in the production of synthetic polymers. Through fermentation processes, starch can form biopolymers or it can be hydrolyzed to form monomers or oligomers. Original starch and modified starch play a very important role in the food industry, they modify the physical properties of food products such as sauces, soups, or meat products [9], gel formation, film formation, and moisture retention. Therefore, in the food industry, starch is used in its granular form as a thickener and adhesive, and in hydrolyzed form as a sweetener. Due to the emergence of research on bioplastics and the outstanding properties of starch, this material has become one of the best options for use in the preparation of packaging films. Thermoplastic starch is prepared by adding a plasticizer to the main starch during its processing. The plasticizers used are: fructose, sorbitol, maltitol, ethanolamine, formaldehyde, water, and glycerol. However, in most studies, water and glycerol have been introduced as the best emollients. Glycerol is usually used in a concentration of 25-30% by weight. Starch-based plastics are complex combinations of starch with compatible plastics such as PLA, PBAT, PBS, PCL, and PHA. Mixing starch with plastics improves water resistance, processing properties, and mechanical properties. Essential oils contain a wide spectrum that can be recognized based on their aromatic compounds. Types of essential oils include azadirachta indicator, lavander angustifolia, thymey vulgaris, eucalyptus globulus, cinnamomum zeylanicum, syzygium aromaticum, citrus limonum, melaleuca alternifolia, and brassica nigra [10-12]. These volatile compounds are responsible for controlling microbial growth and food preservation. For example, neem essential oil is a volatile mixture extracted from the seeds of the neem tree. It (neem plant) has an unpleasant smell of sulfur and garlic. A study has shown that neem essential oil significantly improves the antibacterial activity of polyethylene terephthalate polyester [12, 13]. Lavender essential oil is produced from a plant called lavender through steam distillation. This type of oil has several chemical compounds, including linalyl acetate, linalool, lavandolol, lavandolyl acetate, camphor, etc. [10]. In a research, lavender essential oil was used in furcellaran starch films (a type of gum extracted from Danish seaweed) as gelatin to test their antioxidant, antimicrobial, and physical properties. The results showed that different concentrations (2, 4, and 6%) of lavender essential oil in the film have positive and negative effects on its physical properties. At the same time, the antioxidant and antimicrobial ability is significantly improved and leads to long-term shelf life of packaged food [14].

Food packaging plays a vital role in protecting food products from environmental factors such as ultraviolet rays, oxygen, water vapor, pressure, and heat. It also helps to improve food safety and long-term shelf life by protecting against chemical and microbiological contamination. There are several packaging technologies that help preserve the quality of foods. More innovative approaches such as active packaging are outperforming traditional packaging technologies due to their positive effects in solving environmental problems and increasing consumer acceptance. Although active packaging can contain synthetic additives, there is a growing interest in using bioactive compounds such as essential oils in biodegradable materials for active food packaging. Essential oils in bioactive compounds are suitable for active packaging due to their ability to inhibit the growth of food pathogens and preserve food products [15]. The current applications of essential oils in active food packaging include their use in the form of films and coatings used on different food groups such as fruits, vegetables, fish, meat products, milk and dairy products, bread, and baked foods. The antibacterial potential of aqueous extracts of black pepper, mint leaves, anise, and coriander against 176 bacteria belonging to 12 various genera were determined by disk diffusion method in 2006. The results showed that the aqueous extract of black pepper had the highest antibacterial activity and its antibacterial activity was 75%. Anise extract also had 18.1% antibacterial activity (the maximum antimicrobial activity against micrococcus roseus).

Experimental

Materials and Methods

The starch used in this research was modified corn starch, which was purchased from Pars Avar Company of Science and Technology Park of Semnan University. Cellulose nanofibers (CNF) were purchased from Iranian Nano Materials Company - Iran, which has a white appearance and was prepared in width dimensions of 20 to 50 nm, length dimensions of 20 to 180 micrometers, with a purity of 99.3%, a specific surface area of 31 m^2/g, and a density of 1 g/m^3.

Lemon essential oil was purchased from Barij Essential Pharmaceutical Company and

Merck company glycerol was purchased as a plasticizer.

Preparation of filmed composites

Films of modified starch and cellulose nanofibers were prepared using the casting method (solvent casting method). First, 2 grams of modified starch was dissolved in 60 ml of distilled water and heated for 20 minutes on a magnetic stirrer at 40 °C to 80 °C at a speed of 800 rpm, and then allowed to form a gel. Thereafter, the formed gel is allowed to cool and a fixed amount of glycerol (0.5 mL) was added as a softener to prevent film fragility and cellulose nanofibers were added with a fixed percentage of 0.5 mL (Table 1). Next, a certain percentage of citrus essential oils was added to the starch solution and cellulose nanofibers, each of them was treated together and alone for 15 minutes with a magnetic stirrer and finally poured onto petri dishes with size 19-100 mm, and then the remaining solvent was evaporated for 24 hours at ambient temperature, and after that, in laboratory conditions, the films were exfoliated and finally stored in silica gel. Two microorganisms *Escherichia coli* (*Escherichia Coli* O157:H7 ATCC and 25922) and *Staphylococcus aureus* (*Staphylococcus aureus* ATCC 29213) were used in this research.

Table 1. Composition of obtained starch, cellulose nanofibers, and citrus essential oils

Code	Starch (g)	Cellulose nanofibers (mL)	Glycerol (mL)	Citruse essential (mL)
SNE-1	2	0	0.5	0
SNE-2	2	0.5	0.5	0
SNE- 3	2	0.5	0.5	0.3
SNE-4	2	0.5	0.5	0.5
SNE-5	2	0.5	0.5	0.7
SNE-6	2	0.5	0.5	0.9

Concentration test

With a digital micrometer (China), the films thickness was determined with an accuracy of 0.001 mm at 3 different points which were selected randomly. The average of these points was used to determine other physical and mechanical properties of the films.

Thermal properties using differential scanning calorimetry test

Thermal properties were evaluated by differential scanning calorimetry. An empty container was used as a reference. Samples with an approximate weight of 0.7 g were scanned at a heating rate of 10 °C/min in the temperature range of 0 °C to 180 °C. The glass transition temperature, melting temperature, melting enthalpy ΔH_m, and crystallization percentage X% were measured from the resulting thermos gram.

X-ray diffraction test

The X-ray diffraction pattern was obtained using (ASENWARE.AW-XDM300) diffractometer. A 40 KW accelerating voltage and a 30 mA current were applied using Teta-Teta radiation. Diffraction curves were obtained in the range of scattering angles (2θ) from 0.5 to 160° with a speed of 1° per minute and with a step of 0.05 and a wavelength of 1.54184.

Infrared spectroscopy test

Infrared spectroscopy was used to investigate the changes of functional groups in the samples. Accordingly, FT-IR spectrum was prepared in the wave number range of 450-4000 cm^{-1} using Meager UV9200 infrared spectrometer. All covalently bonded compounds, including organic and inorganic substances, absorb different frequencies of electromagnetic radiation in the infrared area. This method is a powerful and developed method for identifying the structures of molecules, that according to the position of the peaks as well as their intensity, it is possible to understand the diversity of bonds in the structure of the prepared compound. Infrared spectroscopy was used for qualitatively identify the samples, the type of functional groups, and bonds in molecules. During this experiment, the samples were placed in fixed containers, and then placed inside the spectrophotometer and the spectrum of each sample was obtained in the wave number range of 450-4000 cm^{-1}, and by referring to the relevant tables which show the vibration position of different bonds, the wavelength or wave number of the groups and bonds were identified.

Studying microstructure using scanning electron microscope test

Electron microscope images (Philips-Xl30, North America) of the cross-sectional surface of the samples were prepared in order to investigate the effect of adding nanoparticles on the microstructure of the produced nanocomposites and their distribution and also how they interact with the polymer substrate. In preparation of the samples for cross-sectional imaging, first, due to the non-conductivity of the models, a gold coating with a length of 2 to 3 nm was done on all of them to make them conductive, and then the sample was placed on a sputter with carbon paste. The sample was placed inside the machine to be vacuumed. A beam is incident on it, a return electron and a secondary electron at an angle of 37°. Next, these electrons are converted into signals in the device.

Statistical analysis

Experiments were randomly repeated three times. The data obtained from different tests on the samples (starch, cellulose nanofibers, and citric acid) were recorded in Excel software, and SPSS software version 14 was used for statistical analysis of the data. In addition, the P-value was statistically considered less than 0.05.

Results and Discussion

All the samples were placed in the device with the size of 1×3 cm. FT-IR was used to study the material's chemical structure after chemical modification. Stretching vibration of the free hydroxyl groups (-OH) in the nano cellulose molecule and C-H stretching can be seen at 2900 cm^{-1} and 3289 cm^{-1}, respectively. The FT-IR spectra of the starch and CNC are almost identical (Figure 1). The comparison the spectra of starch and starch/cellulose nanofibers films showed new peaks. As reported by Tang *et al.* [14], the rise at 1486.25 cm^{-1} is probably related to the water absorbed by the nano cellulose segment [16] and the absorbed peaks at 580 cm^{-1} and 1162 cm^{-1} are associated with C-O-C asymmetric stretching and alcoholic hydroxyl groups, respectively. A peak was observed at 2151 cm^{-1}, indicating the presence of bonded acetyl groups. For instance, the stretch correlated to the carbonyl groups C=O at 1646 cm^{-1} and connected methyl groups -(C=O)-CH_3 at 1369 cm^{-1} and the time related to acetyl groups C-O in at 1242 cm^{-1} were generated [17]. Ether bonds are formed between the primary alcohol of starch-containing cellulose nanofibers, which leads to a decrease in hydrophobicity. As a result, it is consistent with the experiment conducted by Mohammad *et al.* [9].

Thermal properties

Thermal properties of polymers and nanocomposites are fundamental in relation to flexible food packaging are presented in Table 2 and Figure 2. This study evaluated the thermal properties of cellulose nanofibers and starch-containing citric acid in terms of different parameters such as glass transition temperature, melting temperature, melting enthalpy ΔH_m, and crystallization percentage X%.

The data in the table indicate that T_g and T_m of pure starch (SNE-1) are 58.14 °C and 86.5 °C, respectively. The addition of cellulose nanofibers to refined starch (SNE-2) led to an increase in the T_g. In other words, this nanoparticle improved the T_g of this polymer by 3.53% (the low T_g is one of the main limitations in thermal processes). The changes in T_g, T_m, and crystallization are shown in the following figures. To have a crystalline structure, polymer compounds should have enough time to develop crystals, which is done before they are completely solidified in the manufacturing process.

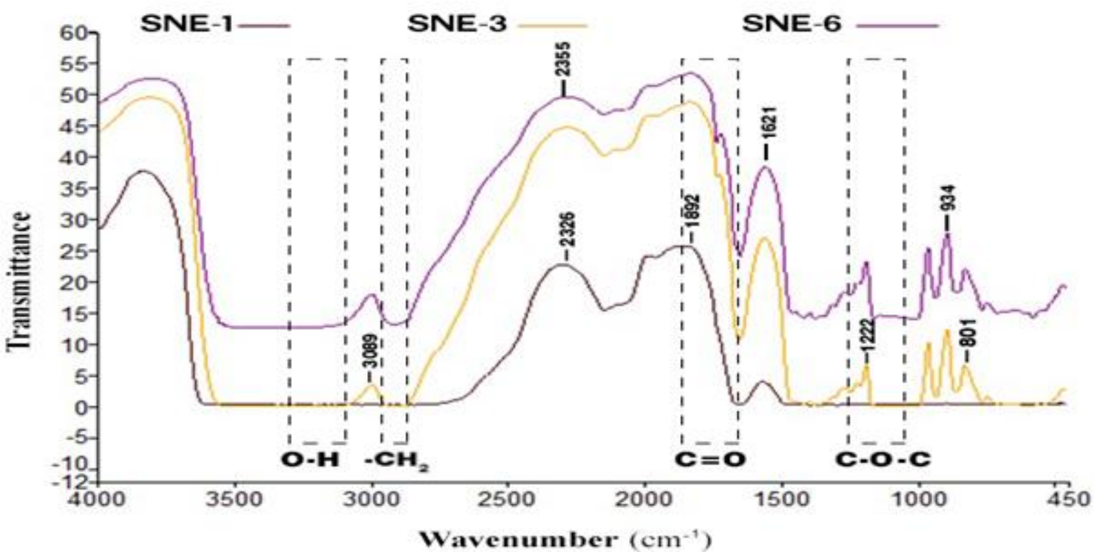


Figure 1. FT-IR spectra for cellulose nanofibers films with starch and Citrus essential oils (SNE-1, SNE-3, SNE-6)

Table 2. DSC results obtained from starch and cellulose nanofibers films containing citrus essential oils (together and alone)

Code	χ%	H_m ($\frac{J}{g}$)	T_m (°C)	T_g (°C)
SNE-1	14.29	4.013	86.56	58.14
SNE-2	19.44	7.379	97.05	61.47
SNE-3	21.55	7.807	94.21	61.21
SNE-4	24.19	8.264	95.11	61.89
SNE-5	25.63	8.572	95.73	61.96
SNE-6	26.59	8.934	95.89	62.03

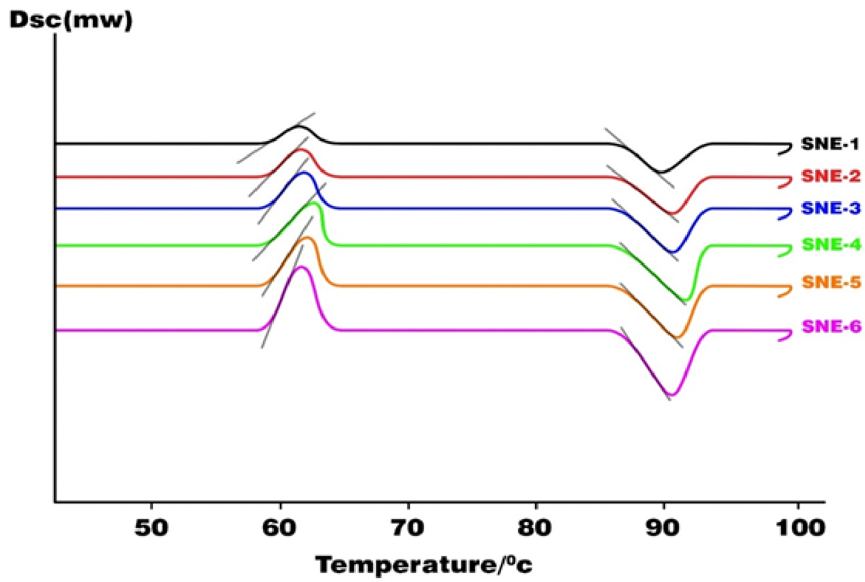


Figure 2. DSC curves of starch and cellulose nanofibers films containing Citrus essential oils

Accordingly, some researchers believe that the producing nanocomposites by the solution method do not lead to a high percentage of crystallization due to the fast evaporation of the solvent and chain solidification. It should be noted that the crystallization process that occurs in polymers due to aging should not be overlooked.

Color

The physical properties such as thickness and color parameters of the produced sample are given in Table 3. Maintaining the transparency of nanocomposites compared to the primary polymer despite nanoparticle incorporations is one of the parameters that must be considered when preparing nanocomposites. The results show that adding cellulose nanofibers in different amounts has little effect on the thickness of the films. This may be due to the excellent dispersion of cellulose nanofibers in both cases. However, a significant increase in viscosity was observed in the SNE-3 sample, suggesting the presence of nanofibers between the matrix polymer strands and the film's surface (a thin layer). The b* index (the yellowness degree of the film) has increased significantly with the increase of essential oil concentration in the studied films, which is consistent with the results of Shavisi *et al.* [18]. In the starch samples with cellulose nanofibers, a change was observed in the increase of a* and b* factors whilst L* factor showed a decreasing trend. In the packaging industry, packaging coloration (film) is needed due to the primary characteristics of the production of new materials. It has a significant effect on its acceptance by the consumer. L*, a*, and b* factors substantially increased the brightness or whiteness of each sample with the increase of raw material. It should be noted that the absolute limit of green and yellow colors is 70 and -80, respectively. Therefore, it should not be expected that the samples' appearance clearly shows these colors' occurrence. According to the present study, citric acid with cellulose nanofibers (alone and together) had no significant effect on the thickness of the starch layer.

Table 3. Color properties of cellulose nanofibers (N), starch (S), and citrus essential oils (E) film

Code	Thickness (µm)	Color factors		
		b*	a*	L*
SNE-1	87.2±20.07	-3.0±958.08a	-4.0±988.06a	18.1±08.26a
SNE-2	87.4±45.03	2.0±730.05b	-3.0±814.02b	79.0±78.89b
SNE-3	88.3±09.01	3.0±670.01b	-3.0±087.03a	78.1±78.56c
SNE-4	87.7±06.24	3.0±698.04c	-4.0±135.09a	78.1±80.69a
SNE-5	88.5±06.04	4.0±896.02ab	-4.0±636.05c	77.2±04.02a
SNE-6	88.7±46.64	5.0±542.05a	-4.0±734.45a	77.0±12.52a

(The lowercase letter and the uppercase letter indicate the difference in columns and rows, respectively)

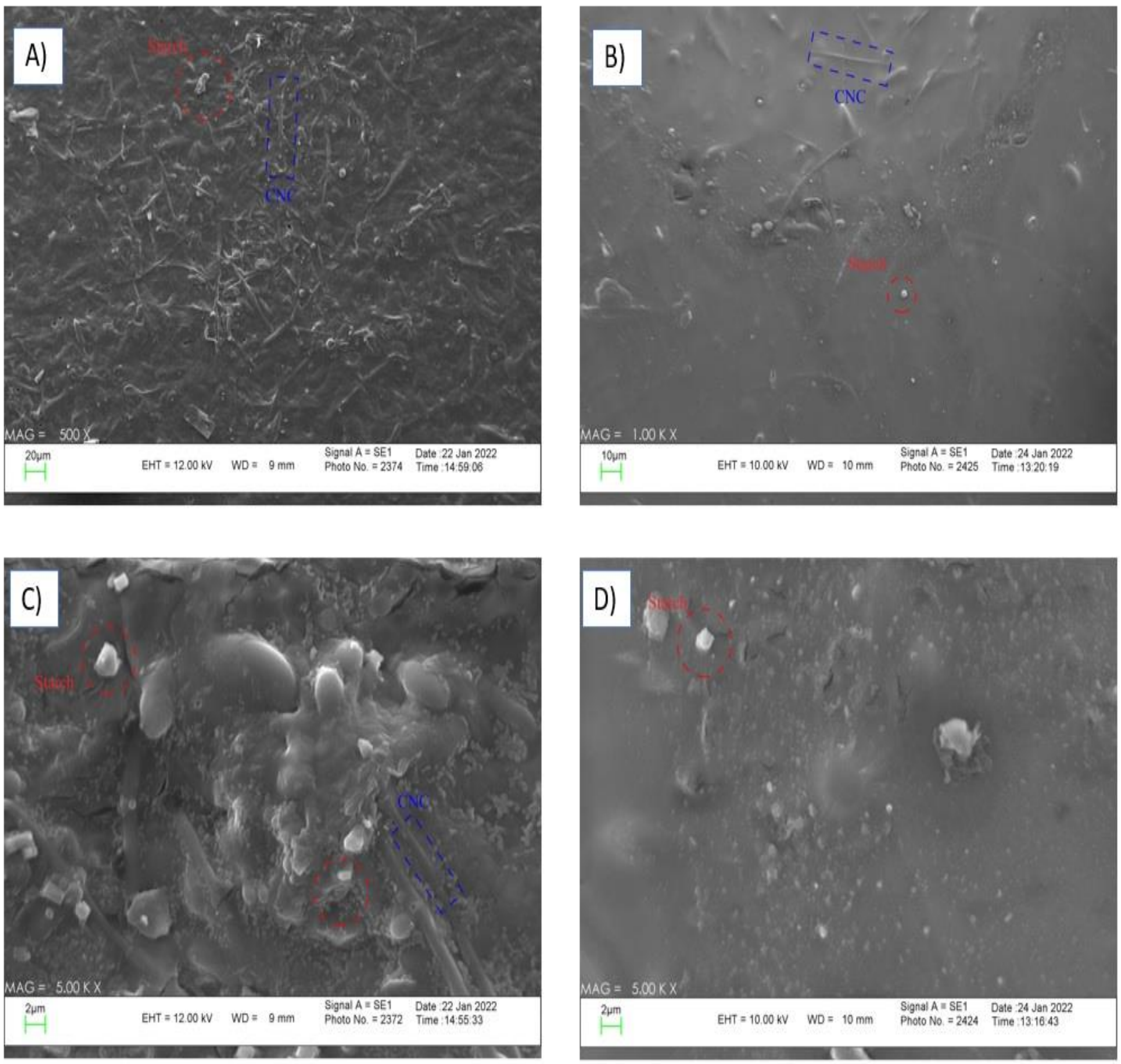


Figure 3. SEM images of the cross-sectional morphologies of foamed composites of the starch and cellulose nanofibers films containing Citrus essential oils, SNE-5(A), SNE-4(B), SNE-3(C), and SNE-1(D)

SEM

The SEM image of starch with cellulose nanofibers and citric acid is depicted in Figure 3. It can be seen that by adding cellulose nanofibers and citric acid, the cracks and pores have been filled, which has resulted in twisting at the location of the pores. This phenomenon has been able to block the path of penetrating molecules well. On the other hand, the cellulose nanofibers are perfectly surrounded by the starch substrate, which indicates the excellent compatibility of cellulose nanofibers with starch. A proper investigation of the images and the results of other tests show an acceptable agreement between them.

XRD

The prepared nanocomposites can form intercalate or exfoliate structure according to the type of incorporated nanoparticle and the manufacturing process (the ability to fill them in the polymer substrate). Therefore, the XRD test is precious to detect these structures and study the nanocomposites' crystalline behaviour. The characterization can be done based on the nanoparticle layer's shape, position, and distance. Cellulose nanofibers were added to the starch matrix as reinforcement. This compound is crystalline and its XRD pattern in Figure 4 demonstrates the presence of a strong peak at 19.29. Regardless of the results of other tests, these results indicate that the cellulose nanofibers have the minimum contact with the starch. Penetration of cellulose nanofibers into the layers can be caused by the nature of cellulose nanofibers and their manufacturing process, which leads to their complete dispersion in the matrix.

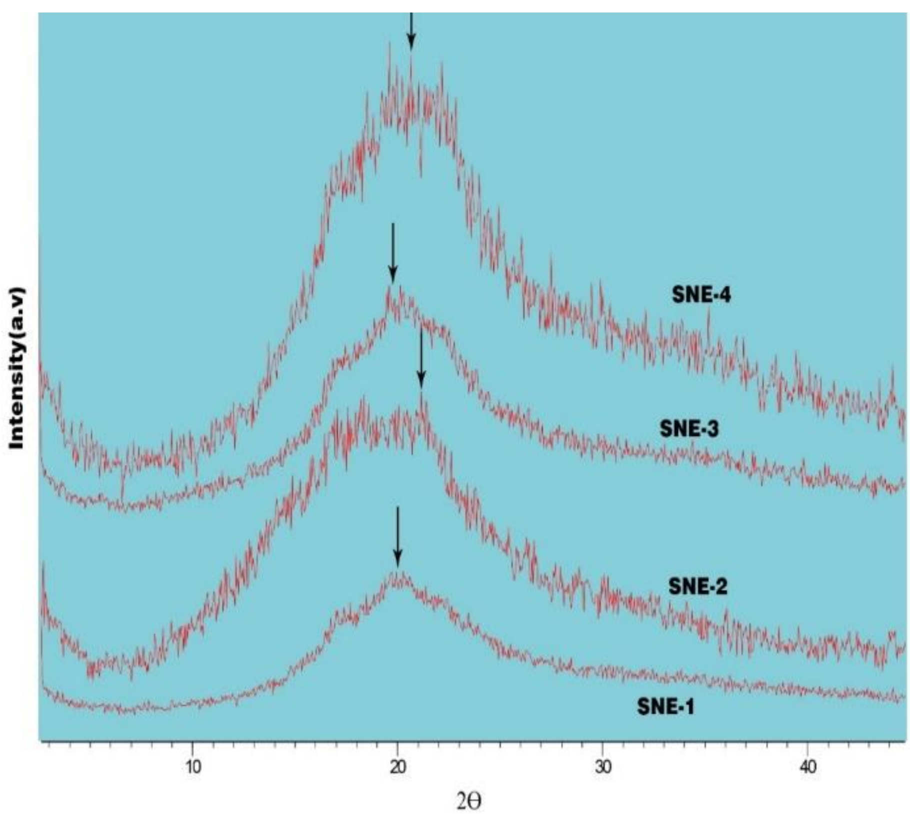


Figure 4. XRD pattern of starch, cellulose nanofibers films containing citrus essential oils

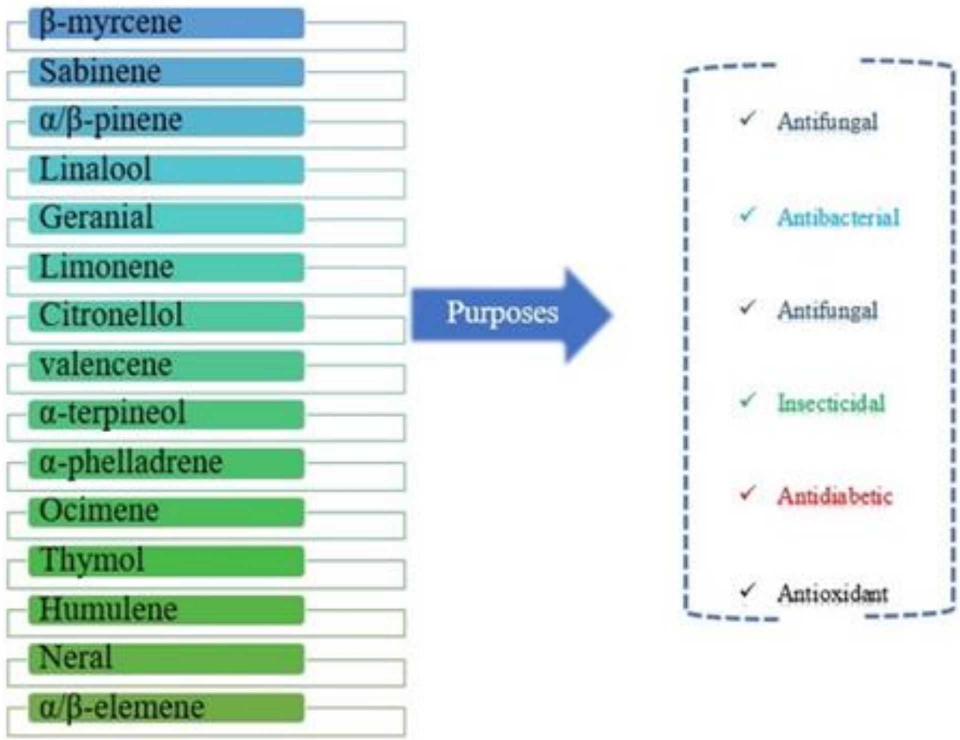


Figure 5. Citrus essential oils and their various purposes

Investigation of the antibacterial effects of films by the disk diffusion method

Escherichia coli and *Staphylococcus aureus* bacteria were purchased as lyophilized vials from the National enter for Genetic and Biological Resources of Iran. The lyophilized ampoules of bacteria were initially transferred to nutrient broth liquid as a culture medium under open aseptic conditions and hence were incubated for 24 hours at 37 °C, and then to ensure the purity of the bacteria, the nutrient broth medium was cultured linearly on the nutrient agar culture medium, and then incubated for 48 hours at 37 °C. To evaluate the antimicrobial effects, a 24-hour culture was prepared for each test. A loop was taken from the white colony and inoculated into the liquid nutrient broth (24-hour culture medium) 24 hours before each test.

The turbidity of the prepared microbial suspension was adjusted to about 1.5×10^{8} $^{cfu}/_{ml}$ by a 0.5 McFarland standard solution. 20 µl of this solution was cultured on the plate surface. In the next step, films with different percentages were placed on the culture surface using sterile forceps and fixed on the culture medium with a little pressure. Petri dishes were set at 37 °C for 24 hours, and then a ruler and Digitizer software were used to measure and analysis digital photos. The difference between the diameter of the formed halos and the discs diameter was considered as an indicator of antimicrobial activity (Figure 6). There was no antimicrobial activity in cases where a halo was not included [19]. The data in Table 4 lists that the antimicrobial effect of starch and cellulose nanofibers films containing citric acid increased significantly ($P>0.05$) with an increase in essence concentration. In contrast, cellulose nanofibers films without citric acid did not display any antimicrobial effects against the studied micro-organisms (P>0.05).

The enormous non-growth halo was related to *Staphylococcus aureus* with a diameter of 25.76 mm, which was identified as the most sensitive bacteria to the antimicrobial film-containing essential oils. CEOs exhibit antioxidant, antidiabetic, insecticidal, antifungal, and antibacterial properties (Figure 5). In this regard, among the plant essential oils, CEOs have drawn more attention because of their broad spectrum insecticidal, antibacterial, and antifungal properties along with their high yields, aromas, and flavours. In addition, CEOs have wide applications in food formulations, packaging, and preservation to ensure food quality and safety.

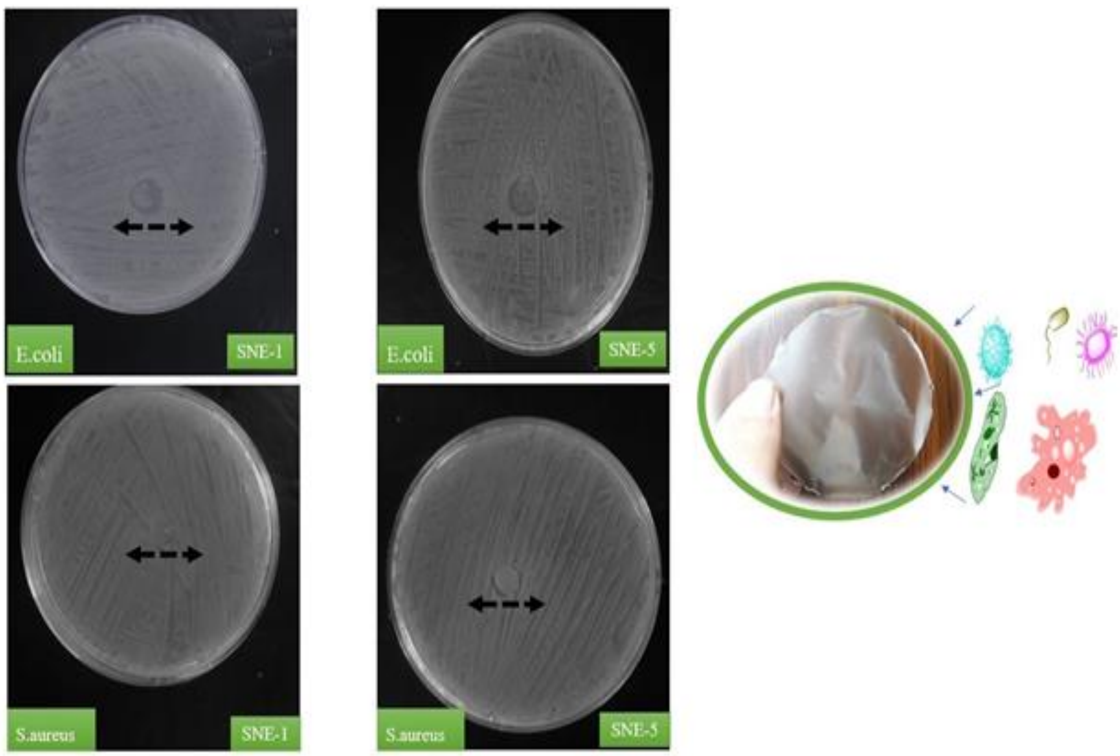


Figure 6. Disc diffusion antibiotic sensitivity test against *Staphylococcus aureus* and *Escherichia coli* (starch and cellulose nanofibers films containing Citrus essential oils)

Table 4. Mean and standard deviation of non-growth halo diameter (mm) around starch and cellulose nanofibers films containing various percentages of citrus essential oils

Microorganisms	SNE-3	SNE-4	SNE-5	SNE-6
Escherichia coli	15/36 ± 0/15 aB	16/51 ± 0/29 aB	16/89 ± 0/66 bC	17/58 ± 0/09 aB
Staphylococcus aureus	16/06 ± 0/22 aB	19/96 ± 0/35 aB	22/31 ± 0/44 aB	25/76 ± 0/25 aB

(The lowercase letter and the uppercase letter indicate the difference in columns and rows, respectively)

Several researchers have conducted extensive studies on the antibacterial properties of EOs and their compounds. Although the mechanism of action of EO components has been clearly explained in many previous works, thorough knowledge about most compounds and their mechanical properties is still lacking.

Conclusion

Concerning the antimicrobial effects of the studied films, it can be stated that starch and cellulose nanofibers films containing citric acid can be used in food packaging due to increased shelf life and reduced food waste. On the other hand, due to the biodegradable nature of these coatings, it is also important to reduce environmental pollution. Examining the surface structure of lemon essential oil, cellulose nanofibers, and starch on the surface of SNE-4 was determined as the best performance in this research.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the article and agreed to be responsible for all aspects of this work.

Orcid

Omid Daliri Shamsabadi
https://orcid.org/0000-0002-6473-2273

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