

Designing active chitosan-based biofilms loaded with the mixture of herbal essential oils and reinforced with nanofibers: Their effects on the shelf life and sensory properties of red meat

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Abstract

Background: Designing the antimicrobial food packaging is needed for food protection and quality maintenance. In this research, active packaging based on chitosan biopolymer with antimicrobial properties has been prepared to investigate its effects on shelf life and sensory properties of red meat. *Origanum vulgare subsp. gracile* essential oil (GEO) and *Carum copticum* essential oil (CEO) compounds (as an antioxidant/antimicrobial substances) from the packaging material into red meat.

Results: According to the obtained results, it was found that the highest percentage of organic compounds identified in *Carum copticum* EO were related to carvacrol (43.94%), gamma-terpinene (26.86%), m-cymene (24.37%) and alpha-pinene (41.2%). The results showed that active films containing different ratios of EOs had high antimicrobial activity against *E. coli* and *B. cereus* bacteria, which were reduced by adding CNF and LCNF due to the role of controlling release of EO compounds.

Conclusion: Microbial evaluation of red meat pieces also showed that the use of edible film coating containing EO significantly reduced the growth rate of microorganisms compared to the uncoated sample. The evaluation of sensory properties of meat samples also showed that the nanocomposite film containing EO increased the shelf life of red meat for more than 20 days at refrigerator temperature due to its antimicrobial properties and favorable aroma.

Introduction

Edible films are defined as thin and continuous layers of edible materials that are placed between food ingredients to create a barrier layer against mass transfer (1). The ingredients of food coating agents can be divided into two categories of water-soluble polysaccharides or fats. Suitable polysaccharides include cellulose derivatives, alginate, pectin, starch, chitosan and other polysaccharides and suitable fats include waxes, acylglycerols and fatty acids (2, 3). After cellulose, chitin and chitosan are the most abundant polymers in the nature. So far, more than 300 different sources of chitin, including marine invertebrates, fungi, bacteria, algae, plants, diatoms, yeasts and insects have been evaluated. Marine organisms are the most important source of chitin and chitosan production, and currently shrimp shells and crab shells are the main sources for chitin and chitosan extraction (4). Chitin and chitosan are widely used in various industries, especially packaging, food industry and biotechnology due to their properties such as high absorbency, non-toxic and non-allergenic, antiviral and antibacterial properties, high expansion and elasticity properties, biodegradability, high ability to convert into various substances and derivatives and etc (5). Chitosan is the only cationic polysaccharide that is prepared with deacetylation of chitin by chemical, enzymatic and microbiological methods. This biopolymer is made of repeating units of beta-(1–4)-2-amino-digluco (6).

Nanocomposite refers to a special category of composites that at least one of their components is on the nano scale. In polymer nanocomposites, there is a better interaction between the polymer matrix and the filler than in conventional composites. The uniform distribution of nanoparticles in the polymer matrix

increases the contact surface of the matrix and nanoparticles, which results in the improvement of mechanical, thermal, and barrier properties (7). Cellulose in the form of nanocrystals has high tensile strength and Young's modulus and is considered a good reinforcing material for various composite materials (8). The reinforcing ability of cellulose nanocrystals is due to their lateral area and good mechanical properties. Since cellulose nanofibers prepared from plant sources have the ability to be extracted in the form of thinner fibers than bacterial cellulose, many researchers are investigating the extraction of nanofibers from wood and other plant fibers (9).

Plant EOs have a natural base, so their chemical compounds are influenced by environmental and hereditary factors. Factors such as the type of species and subspecies, geographical location, harvest time and the used part of the plant determine the composition of crude plant EOs (10). Among the compounds of EOs, terpenoid compounds are responsible for the antimicrobial activities (11). Available evidence shows that even minor compounds can exert significant antimicrobial properties through synergistic effects with other compounds (12). The plant *Origanum vulgare* belongs to the mint family, which contains 1-1.5% EO, whose main compositions are phenolic compounds such as carvacrol and thymol (13). Researches have shown that the thymol, carvacrol and cinnamaldehyde present in *Origanum vulgare*, cinnamon and thyme EOs exhibited the antibacterial effects against *Escherichia coli* O157 and *Salmonella typhimurium* (14). *Carum copticum* is an annual plant of the umbrellae family that grows in Iran, India and Egypt with small white flowers and light brown seeds. *Carum copticum* is used orally as an analgesic, anti-asthmatic, anti-nausea and expectorant and topically in the treatment of rheumatic pains (15). Researches have shown that the aqueous extract of this plant is very effective in treating children's colds. The highest amount of plant EO is found in its fruit (seed) and its amount varies between 2–5% according to genetic and environmental conditions (16). In this research, active packaging based on chitosan biopolymer with antimicrobial properties has been prepared to investigate its effects on shelf life and sensory properties of red meat. Lignocellulose nanofibers (LCNF) and cellulose nanofibers (CNF) were used to enhance the properties of biopolymer and also to control the release of *Origanum vulgare* and *Carum copticum* EO compounds (as antioxidant/antimicrobial substances) from the packaging material into red meat.

Material

Chitosan with medium molecular weight (KD 190–310) and deacetylation degree of 75–85% was purchased from Sigma Aldrich (USA). Cellulose nanofiber with a dry matter content of 2.5% was purchased from Nanovin Polymer Company (Sari, Iran). Nanofibrolignocellulose (containing lignin, cellulose and hemicellulose) with a dry matter content of 2.5% was purchased from Nanovin Polymer Company (Sari, Iran). *Origanum vulgare* plant leaves and seeds were prepared for extracting EO from the Department of Horticulture, Faculty of Agriculture, Urmia University. Other ingredients include glycerol with a purity of 99%, ethanol with a purity of 96%, calcium sulfate and potassium sulfate were purchased from Sharlou Co. Acetic acid, Tween 80 and PCA (for total bacteria count), SSA (for *Salmonella* culture),

VRBA (for coliform culture), EMBA (for *Escherichia coli* culture) and SDA (for mold and yeast culture) were prepared from Merck, Germany. Red meat was prepared from a reliable butcher in the city.

Material and Methods

EOs extraction

To produce the pure EO of *Origanum vulgare* and *Carum copticum*, an EO extractor (Clevenger) was used. Then, the obtained EO was stored in dark glasses at the refrigerator temperature until the test.

Film production

Chitosan with an average molecular weight of 2% by w/v was dissolved in 1% acetic acid and placed on a magnetic hot plate at 1200 rpm for 2 hours at a temperature of $45 \pm 1.5^{\circ}\text{C}$ until the solution obtained the homogeneity. Then, glycerol (0.8 g) was added to the mixture as a softener and the stirring continued on the hot plate for 15 minutes (control films). For the production of active composite films, 5% by weight of chitosan, the GEO and CEO, was added to the mixture separately or in combination (based on the experimental design) and immediately 0.2% of EO, emulsifier Tween 80 was added to the mixture. Next, the mixture was stirred for 1.5 hours by a magnetic stirrer until a milky and uniform emulsion was formed. Then the final mixture was spread in plates with a diameter of 10 cm (33 g). The plates were placed in an oven at a temperature of $30 \pm 1^{\circ}\text{C}$ for 72 hours to dry the films completely. To produce active composite films containing nanofibers (active bionanocomposite), first 4% by weight of chitosan, cellulose nanofiber gel or lignocellulose is spread in distilled water, then to spread evenly in the film by ultrasound device (ROHS Digital-DSA100-SK2 South Korea). It was subjected to ultrasonic waves with an intensity of 40 KHz (100 W) for 30 minutes (17). The produced films had an average thickness of $120 \pm 5 \mu\text{m}$, which was measured by a manual micrometer (Alton M820-25, Nanjing, Jiangsu, China) with an accuracy of 0.01 micron (6).

Analyzing the chemical composition of EOs

EO samples were analyzed using a Varian 3400 model gas chromatography device connected to a mass spectrometer (GC-MS) and equipped with an HP-5MS capillary column (30 m long, 0.25 mm diameter, and $25 \mu\text{m}$ inner layer thickness). The carrier gas was Helium with a flow of 1.5 ml/min and the split ratio of the sample was 1:10. The temperature program of the column was set from 60 to 250°C at a speed of $3^{\circ}\text{C}/\text{min}$ and it was kept at a constant temperature of 250°C for 10 minutes as a post run. The temperature of injection and detection was set to 260°C . The ionization voltage of the mass spectrum was equal to 70 electron volts and the temperature of the ionization source was equal to 250°C . The identification of EO components was performed by comparing their mass spectrum with the spectral bank and comparing their inhibition coefficients with reference values (18).

Antimicrobial property of the film produced on the microbial flora of red meat

First, the meat was divided into 30-gram pieces to be covered with the produced films, then they were divided into 4 groups.: uncoated (control), coated meat made with pure chitosan film, chitosan film containing 5% GEO and CEO and samples coated with film containing 5% EO and LCNF nanofiber (optimal film). Then the coated meats were kept in the refrigerator at 5°C for 20 days (Fig. 1). Meats were sampled for microbial tests every 5 days. To prepare the initial meat suspension (dilution 1–10), 10 gr of meat samples were homogenized with 90 ml of Ringer's solution in a Stumker machine (Interscience Ltd., France) for 2 mins. Then, decimal dilutions were prepared from the resulting suspension (19).

Sensory evaluation of red meat

The purpose of this test was to determine the overall acceptance of red meat covered with film samples by panelists during five time periods of 0, 5, 10, 15 and 20 days. For this purpose, 15 students and employees of the Faculty of Agriculture of Urmia University were asked to conduct the test. The panelists were asked to check the samples in terms of odor parameters (fresh meat smell to rotten smell), appearance color (light to dark pink) and overall acceptance (completely acceptable to completely unacceptable). A 9-point hedonic scoring system (1 is very bad and 9 is very good) was used to evaluate the samples (20).

Statistical Analysis

A combined design with three factors including GEO concentration (0–5%) and CEO concentration (0–5%) and nanofibers type (Blank, LCNF and CNF) was used to investigate the effects of produced active films on the shelf life of red meat. Experiments were designed and results were analyzed by Design Expert version 13 software.

Results and discussion

Analysis of EO compounds

The extraction efficiency of EO from seeds of the plant *Carum copticum* was 2.9% in this research. The main chemical compounds of the EO and their amounts are shown in Table 1. According to the obtained results, it was found that the most dominant organic compounds identified in *Carum copticum* EO include carvacrol (43.94%), gamma-terpinene (26.86%), m-cymene (24.37%) and alpha-pinene (41.2%).

The EO extracted from plants varies in terms of quantity, quality and type of chemical compounds that constitutes the EO depending on weather conditions, soil composition, plant organ, and age and growth stage of the plant (21). For example, the research conducted by Rasooli et al. (2008) showed that the most dominant compound detected in the EO of *Carum copticum* was p-simene (22), while in other studies gamma-terpinene was the main detected compound in the EO of this plant (15, 16), but in the present research, carvacrol was identified as the dominant chemical compound in the EO of *Carum copticum*. Antioxidant activity of *Carum copticum* EO which was measured as IC₅₀ (concentration that regenerates 50% of free radicals) in different concentrations (0.125, 0.25, 0.5, 1, 2 and 4 l/ml μ) was 1.21

μl/ml. It is clear that the smaller amount of this index indicates the higher antioxidant power of the EO which is due to the presence of compounds such as carvacrol and gamma-terpinene.

Table 1
Chemical composition of the EO obtained from the seeds of the *C. copticum*

Number	Ingredients	Retention time (min)	Content (%)
1	α-Phyllanderene	9.61	0.4
2	α-Pinene	11.4	2.41
3	α-Myrcene	12.06	0.57
4	Cis-4-carene	12.96	0.29
5	m-Cymene	13.39	24.37
6	(E)-3-Caren-2-ol	13.46	0.52
7	γ-Terpinene	14.67	26.86
8	Artemiseole	18.69	0.22
9	Carvacrol	23.69	43.94
Total		-	99.58

Analysis of GEO compounds

Origanum vulgare plant had EO with yellowish green color and extraction efficiency of 1.65%. The chemical compounds of the EO, which were identified and measured by GC/MS, included 20 different types of compounds. The list of identified compounds of GEO is shown in Table 2. According to the obtained results, carvacrol was the largest amount among the components of the EO with 43.49%. Other important components of the EO included gamma-terpinene (17.71%), m-cymene (7.96%), ethyl methylthymol (5.45%) and thymol (3.28%). Few reports have been published on the identification of GEO compounds.

For example, Moradi et al. (2015) reported that the carvacrol, gamma-terpinene, and p-cemen are the main components of the EO of leaves and flowers of *Origanum vulgare* grown in Iran (13), which is consistent with the results of this research. The EO obtained from gracile and virens species of *Origanum vulgare* grown in France was rich in chemical compounds sabinene and germacrane D (23), while the thymol, gamma-terpinene, beta-caryophyllene, alpha-terpinolene, p-cymene and carvacrol have been reported as the main components of the EO of the *Origanum vulgare* plant grown in Turkey (24). All this indicates that there are some differences between the various results which is probably due to different environmental and genetic factors, nutritional status, harvesting time, and also the part of the plant that was used to extract the EO.

Table 2
Chemical composition of the EO obtained from the leaves of *O. vulgare*
ssp. *Gracile*

Row	Ingredients	Retention time (min)	Percent (%)
1	α -Thujene	9.61	1.58
2	α -Pinene	9.82	0.83
3	Camphene	10.34	0.06
4	2-Thujene	11.33	0.08
5	β -Pinene	11.39	0.24
6	3-Octanone	11.97	0.80
7	α -Myrcene	12.05	2.56
8	α -Phellandrene	12.48	0.23
9	cis-4-Carene	12.95	2.47
10	m-Cymene	13.30	7.96
11	(E)-3-Caren-2-ol	13.42	0.31
12	Eucalyptol	13.49	0.23
13	α -trans-Ocimene	13.85	1.16
14	delta-3-Carene	14.24	0.12
15	γ -Terpinene	14.61	17.71
16	cis- α -Terpineol	15.01	0.82
17	p-Menth-1-en-4-ol	19.10	0.47
18	p-Menth-1-en-8-ol	19.69	0.46
19	Thymol methyl ether	21.41	5.45
20	Thymol	23.68	3.28
21	Carvacrol	24.03	49.43
22	Isocaryophyllene	27.21	0.72
23	Germacrene D	30.05	0.22
24	α -Caryophyllene	31.22	2.09
25	Linoleic acid ethyl ester	46.62	0.7
	Total	-	99.98%

Antimicrobial properties of films

Disc diffusion method was used to determine the antimicrobial property of active films and nanocomposite. In the disk diffusion method, the antimicrobial film is placed in a solid culture medium that contains the desired microorganism. After incubation at a certain temperature, the formation of an inhibition zone around the film indicates the release of the antimicrobial agent from the film and thus inhibition of the growth of the desired microorganism. The disk diffusion test method simulates food packaging and shows the performance of the film in contact with contaminated surfaces and the migration of the antimicrobial agent from the film to the food (25). The results of the antimicrobial activity of the active films based on chitosan alone its combination with EOs and nanofibers are shown as inhibition zone diameter on the gram-negative bacteria (*E.coli*) and the gram-positive bacteria (*B.cereus*) in Table 3. It is clear that the pure chitosan film was effective against both types of the studied bacteria with the inhibition zone diameter of 3.74 and 5.08 mm for *E.coli* and *B.cereus* bacteria, respectively.

Table 3
Inhibition zone diameter of active films and active chitosan films containing different ratios of CEO and GEO in combination with organic nanofibers by disk diffusion method

	GEO	CEO	Nanofibers	(mm)Inhibition diameter zone	
Rows				<i>B. cereus</i>	<i>E. coli</i>
Control	0	0	0	5.08	3.74
1	0	5	LCNF	15.38	11.68
2	2.5	2.5	CNF	15.23	12.1
3	3.75	1.25	CNF	14.16	10.85
4	0	5	CNF	17.97	13.88
5	1.25	3.75	CNF	16.5	13.08
6	5	0	CNF	13.34	9.26
7	2.5	2.5	Blank*	18.78	14.4
8	2.5	2.5	LCNF	14.26	10.98
9	5	0	LCNF	12.89	8.78
10	5	0	Blank*	14.08	11.22
11	0	5	LCNF	15.5	11.75
12	5	0	LCNF	12.98	8.37
13	1.25	3.75	LCNF	14.95	11.03
14	5	0	Blank*	13.73	11.29
15	3.75	1.25	LCNF	13.75	9.25
16	3.75	1.25	Blank*	15.56	13.05
17	5	0	CNF	13.67	9.45
18	0	5	Blank*	20.78	15.38
19	0	5	Blank*	20.1	15.26
* Films without nanofibers					

Various reports on the antimicrobial activity of chitosan films have been presented in scientific literature. For example, Wang et al. (2011) and Ojagh et al. (2010) reported that there was no significant difference in the inhibition zone diameter for the effect of pure chitosan film on some Gram-negative and Gram-positive bacteria (26, 27), While Genskowsky et al. (2015), Du et al. (2009) and Kim et al. (2011) stated

that pure chitosan films had significant antimicrobial activity on *Bacillus subtilis*, *E. coli* and *S. aureus* bacteria (25, 28, 29). The antimicrobial property of chitosan is due to the positive charge of amine groups, which leads to DNA damage in microorganisms (30). The type of chitosan and especially its degree of acetylation, molecular weight, target organism and environmental conditions significantly affect the antimicrobial activity of chitosan. The environmental conditions which can be considered include pH value, ionic strength and the presence of solutes sensitive to the reaction with chitosan through electrostatic interaction or covalent bonds that can block the reactivity of the active amine group.

The inhibitory effect of films against bacterial growth showed a significant increase by adding 5% of GEO and CEO separately and in combination to the chitosan matrix which is due to the diffusion of the active compounds of the EOs through the agar gel. In this case, the inhibition zone diameter for *E.coli* and *B.cereus* bacteria that were affected by the active film with 5% GEO was measured as 11.22 and 14.08 mm, respectively, while in the case of the active film with 5% CEO, it was 15.38 and 20.78 mm, respectively which are significantly different from the control sample (Table 3). The inhibition zone diameter for the active film with a ratio of 50:50 of the combination of two Eos (5%) for *E.coli* and *B.cereus* bacteria was 14.4 and 18.78 mm, respectively. From the results, it can be concluded that the antimicrobial property of CEO is significantly higher than GEO at constant concentration, while the investigation of the antioxidant properties of these two EOs showed that the inhibitory activity of GEO is about 2.5 times that of CEO. In general, the hydrophobic nature of EOs enables them to react with lipid structures such as bacterial cell membranes, mitochondria and other intracellular compounds, thereby causing disruption of the cell structure, leakage of compounds inside the bacterial cell to the outside, change of charge ionizing the cell, preventing cellular respiration and changing the permeability of the cell membrane and finally leading to the death of the bacterial cell (31, 32).

The results also showed that the antimicrobial effect of nanocomposite films decreased significantly by adding 4% CNF or LCNF organic nanofiber (Table 3). For example, the inhibition zone diameter for *E.coli* and *B.cereus* bacteria was 13.88 and 17.97 mm, respectively in the CNF nanocomposite film with 5% Carum copticum EO while it was 11.68 mm, and 15.38 mm, respectively for LCNF film with 5% Carum copticum EO. These results showed that the nanofibers act as agents to the release control of active compounds and as a result, a smaller amount of these compounds are released from the nanocomposites into the agar gel during 24 sat at 37°C. Also, the hydrogen bonds formed between the carboxyl groups of chitosan and nanofibers (which was confirmed by FTIR test) lead to a greater cohesion of the structure of nanocomposites. In this case, the active ingredients of EOs should take a longer path through the nanocomposite layer to enter the agar gel, which leads to a decrease in the release rate of antimicrobial agents (33). According to the results of Alboofetileh et al. (2014), various factors such as the chemical composition of EOs, the type of bacteria, the properties of the matrix of films, the method and conditions of production of films are effective on the antimicrobial properties of edible films (33).

In general, the results showed that *B.cereus* (gram positive) had less resistance compared to *E. coli* (gram negative) against the antibacterial effect of films containing EO, so that the inhibition zone diameter for

all films was obtained in the range of 5.08 to 20.78 mm for the gram positive bacteria *B. cereus*, but the inhibition zone diameter was 3.74 to 15.38 mm for gram-negative bacteria *E. coli* (Table 3 and Fig. 2). This difference may be due to the fact that Gram-negative bacteria are more resistant to antibacterial agents due to the outer liposaccharide layer that surrounds the peptidoglycan wall of the bacteria (31).

In a similar research, Jafari et al. (2016) investigated the antimicrobial properties of chitosan film and its nanocomposites against *Salmonella Typhimurium* by disk diffusion method and reported that the effect of chitosan nanocomposite films containing silver was significantly different from pure chitosan so that the greatest effect was on CS/Ag film with inhibition zone of 97.86 mm², but the inhibition zone diameter was reduced with the addition of chitin nanofibers, bacterial cellulose nanofibers, and nanoclay which was due to the role of nanoparticles in release control the antimicrobial agent of nanosilver from the chitosan matrix. Meanwhile, bacterial nanofibrocellulose had less ability to reduce the release of silver nanoparticle (34).

The effect of active films on the microbial characteristics of red meat

In this research, active chitosan films containing GEO and CEO were produced and used to cover red meat pieces in order to control the microbial load and improve its sensory properties. The average logarithm count of aerobic mesophilic bacteria and mold and red meat yeast coated with active films as well as the uncoated sample stored at 5°C for 20 days are shown in Table 4. The results of the analysis of variance showed that both the type of treatment and the storage time had a significant effect on the changes in the microbial flora of the red meat samples stored in the refrigerator ($p < 0.05$). In general, the microbial flora increased significantly in all samples ($p < 0.05$) during the storage of meat pieces in cold conditions with the more rate in uncoated sample compared to the coated samples (Table 4).

According to the results, the total count of mesophilic bacteria in the uncoated red meat sample increased from the initial value of 3.34 Log CFU/g to 7.37 Log CFU/g on the 15th day, while the total number of bacteria was not countable on the 20th day of storage. In the case of the samples covered with chitosan films, better results were obtained, so that even after 20 days of keeping the meat pieces in the refrigerator, the total bacteria count was less than the standard limit (10^7 CFU/g), which indicates the remarkable effect of chitosan films in the control of total count bacteria. As shown in Table 4, the pure chitosan film had the least effect, so that at the end of the storage period, the total number of bacteria was equal to Log CFU/g 6.32, but in the case of active chitosan film with 5% GEO/CEO and active chitosan film with combination of 5% GEO/CEO and LCNF, total bacteria count was 4.90 and 5.21 Log CFU/g, respectively on the 20th day of storage.

Table 4

The effect of active chitosan films on the microbiological status (Log CFU/g) of red meat stored for 20 days at 5°C

Microorganism group	treatments	Storage time (days)				
		0	5	10	15	20
<i>Total Count</i>	Control	3.34 ± 0.11i	4.48 ± 0.15f	5.64 ± 0.22c	7.37 ± 0.21a	-
	Chitosan	3.35 ± 0.08hi	4.08 ± 0.18g	5.11 ± 0.14ed	5.31 ± 0.12d	6.32 ± 0.22b
	CH-CGEO*	3.34 ± 0.15i	3.28 ± 0.10i	3.88 ± 0.10g	4.05 ± 0.11g	4.90 ± 0.10e
	CH-CGEO-LCNF**	3.32 ± 0.21i	3.59 ± 0.11h	3.95 ± 0.10g	4.62 ± 0.20f	5.21 ± 0.12d
<i>Salmonella</i>	Control	2.90 ± 0.09g	3.22 ± 0.21ef	3.64 ± 0.11d	6.51 ± 0.18a	-
<i>Shigella (SS)</i>	Chitosan	2.91 ± 0.06g	1.24 ± 0.10k	2.56 ± 0.10h	3.35 ± 0.10e	4.48 ± 0.11b
	CH- CGEO*	2.88 ± 0.15g	0.00 ± 0.00l	1.89 ± 0.09j	2.58 ± 0.14h	3.82 ± 0.10d
	CH-CGEO-LCNF**	2.90 ± 0.10g	1.12 ± 0.09k	2.11 ± 0.07i	3.14 ± 0.09f	4.06 ± 0.20c
<i>Coliforms</i>	Control	2.54 ± 0.22f	3.81 ± 0.10d	5.24 ± 0.10b	6.29 ± 0.25a	-
	Chitosan	2.50 ± 0.17fg	1.28 ± 0.11j	2.17 ± 0.08h	3.09 ± 0.20e	4.25 ± 0.10c
	CH- CGEO*	2.55 ± 0.08f	0.00 ± 0.00k	1.34 ± 0.10j	2.47 ± 0.11fg	3.74 ± 0.08d
	CH-CGEO-LCNF**	2.51 ± 0.12fg	0.00 ± 0.00k	1.88 ± 0.11i	2.31 ± 0.15gh	4.12 ± 0.25c
<i>E.coli</i>	Control	2.00 ± 0.05fg	3.46 ± 0.15d	4.36 ± 0.23b	5.61 ± 0.19a	-
	Chitosan	2.05 ± 0.10f	0.98 ± 0.05i	1.89 ± 0.04fg	2.91 ± 0.09e	4.15 ± 0.17c
	CH- CGEO*	2.02 ± 0.10f	0.00 ± 0.00k	0.71 ± 0.02j	1.51 ± 0.2h	3.11 ± 0.10e
	CH-CGEO-LCNF**	2.00 ± 0.13f	0.00 ± 0.00k	0.51 ± 0.08j	1.80 ± 0.11g	3.59 ± 0.20d

Microorganism group	treatments	Storage time (days)				
		0	5	10	15	20
<i>Yeast and Mold</i>	Control	0.71 ± 0.04i	1.63 ± 0.06g	3.31 ± 0.10d	5.35 ± 0.15b	-
	Chitosan	0.69 ± 0.06i	0.94 ± 0.01hi	1.99 ± 0.120f	3.12 ± 0.12d	5.94 ± 0.23a
	CH-CGEO*	0.70 ± 0.10i	0.00 ± 0.00j	1.12 ± 0.08h	2.79 ± 0.14e	4.74 ± 0.25c
	CH-CGEO-LCNF**	0.69 ± 0.00i	0.00 ± 0.00j	1.59 ± 0.06g	2.61 ± 0.10e	5.28 ± 0.20b

Different letters indicate a significant difference between the means at the $p < 0.05$ level.

* Active chitosan film with 5% GEO/CEO ** Active chitosan film with combination of 5% GEO/CEO and LCNF

Petrou et al. (2012) reported that the total bacterial count of chicken breast meat treated with 1.5% chitosan solution containing oregano EO in combination with modified atmosphere packaging (MAP) was significantly reduced compared to the control sample, and the shelf life of the breast meat was raised to 10 days (35). Sirocchi et al. (2017) also investigated the effect of rosemary EO in combination with different packaging conditions on increasing the shelf life of red meat stored for 20 days at refrigerator temperature. Their results showed that the growth of psychrophilic bacteria, *Pseudomonas* and *Enterobacteriaceae*, was significantly affected by rosemary EO and the amount of oxygen inside the package, so that the best conditions for increasing the shelf life of red meat after 15 days storage was detected in treatments with both rosemary EO and the MAP containing high oxygen (19).

In the case of Salmonella group, the highest number was counted in the uncoated sample, from 2.90 Log CFU/g on the first day to 6.51 Log CFU/g on the 15th day, which indicates an increase of 4 logarithmic cycles in the number of this group of bacteria in meat (Table 4). Meanwhile, the number of salmonella counted in the meat pieces covered with chitosan films even on the 20th day of storage was 2–3 cycles less than the number of this bacteria in the uncoated sample on the 15th day, which indicates the remarkable growth control of this bacteria by chitosan coating. The similar results were also obtained for other microorganisms including coliforms, *Escherichia coli*, mold and yeast during the storage time so that the highest contamination and microbial count was always observed in the uncoated sample. The chitosan coating films especially the active films containing EO was able to significant control the growth of these microorganisms at refrigerator temperature. Also, in terms of growth control for studied microorganisms, there is no significant difference between the chitosan active film with GEO/CEO and the chitosan active film with GEO/CEO-LCNF nanocomposite but a significant difference was observed compared to pure chitosan film and also the control samples (Table 4).

The interesting thing about the activated films containing 5% GEO and CEO was that the number of *Salmonella*, coliform, *Escherichia coli* and mold and yeast was zero on the 5 days of storage. In fact, the EO contained in these films prevented the growth of these bacteria in the first 5 days due to the difference in its concentration between the depth and surface layers of the meat and its faster diffusion. Absorption of water from the meat surface by chitosan films and consequently reducing water activity can also be another factor in preventing the growth of bacteria in the early days. In a similar study, Honarvar et al. (2017) also investigated the effect of thyme EO (*Zataria multiflora*) in polypropylene film coated with CMC on increasing the shelf life of chicken breast meat at 4°C. Their results showed that the total count of bacteria and the population of *Pseudomonas* significantly decreased and the shelf life of breast meat stored in PP/CMC packaging containing 4% thyme EO increased by about 9 days compared to uncoated samples (36). Quesada et al. (2016) also used active antimicrobial packaging containing thyme (*Thymus vulgaris* L) EO based on chitosan to increase the shelf life of ready-to-eat meat (hamburgers) for 4 weeks in cold conditions, and concluded that the mold and yeast population decreased significantly compared to the control sample, but there was no significant difference between treated and control samples in the count of aerobic mesophilic bacteria, lactic acid bacteria and *Enterobacteriaceae* (37).

Hassanzadeh et al. (2017) also investigated the effect of chitosan edible coating (2% solution) containing grape seed extract (0.1%) on the microbial quality and shelf life of chicken breast meat at refrigerator temperature. Their results indicated that the coating and resulted in reducing the count of mesophilic and psychrophilic bacteria increased the shelf life at least 7 days (38). Bazargani et al. (2015) achieved similar results in the use of chitosan film containing pomegranate extract and thyme EO to increase the shelf life of ready-to-cook poultry and chicken meat products at refrigerator temperature, respectively (39).

The effect of active films on sensory properties of red meat

The important sensory changes of meat, especially in oxygen-permeable packaging, include the creation of undesirable changes in its color, smell, and taste, which are the result of bacterial growth and chemical changes (lipid oxidation) and the production of volatile compounds. The extent of these changes in meats is different based on various factors, which ultimately leads to a reduction in the shelf life of meat (40, 41).

The results of sensory properties on red meat samples under different treatments during the storage time by the panelists are shown in Fig. 3. As can be observed, the control and coated samples had acceptable grades in the evaluation of color, smell, and overall acceptability until the 10 days of storage in the refrigerator. On the 15th day of storage, the control sample showed symptom of spoilage as an inappropriate smell and a slimy state due to the fat oxidation and microbial growth, for this reason, it was considered as a weak and unacceptable sample and was removed. Therefore, the shelf life of uncoated red meat at refrigerator temperature was 10 days, so that it has obtained 7.2, 6.3, and 6.4 scores for color, smell, and general acceptability, respectively on the 10th day of storage while this scores decreased to 4.5, 2, and 2.5, respectively on the 15th day of storage. In the case of coated red meat samples, it was obtained low scores but acceptable on the 20th day, so that for all the coated samples, the sensory score

of appearance color, smell, and overall acceptance was higher than 5 (average) (Fig. 3). Therefore, based on sensory observations, coating the red meat pieces with edible chitosan film increased the shelf life of the meat in refrigerator temperature by 20 to 25 days, while the uncoated samples could only be stored for 10 days. According to the results, among the coated samples, the highest sensory score belonged to chitosan active film with GEO/CEO and the chitosan active film with GEO/CEO -LCNF nanocomposite, so that the overall acceptance score of the meat pieces coated with these films was 7 and 2.7, respectively after 20 days of storage in the refrigerator (Fig. 4).

Vasilatos and Savvaidis (2013) investigated the effect of chitosan coating alone and in combination with rosemary EO on increasing the shelf life of turkey meat. Evaluation of their samples indicated a decrease in their sensory scores during 21 days of storage at 2°C, so that the odor and taste scores in the uncoated sample were significantly lower than the treated samples. Finally, they reported that the shelf life was 10, 17–18, more than 21 and more than 21 days for uncoated sample, sample containing rosemary EO and the sample covered with empty chitosan and chitosan containing EO, respectively (42), which is consistent with the results of present study. Banon et al. (2007) and Carpenter et al. (2007) also used chitosan coating containing grape seed extract on beef and pork samples (43, 44), as well as Bazargani-Gilani et al. (2015) and Honarvar et al. (2017) achieved similar results using Thyme EO coated with chitosan and PP/CMC, respectively to preserve chicken meat stored in cold conditions (39, 36).

Conclusion

Different proportions of essential oil of *Origanum vulgare* and especially *Carum copticum* significantly increased the inhibitory activity of chitosan films against *E.coli* and *B.cereus* bacteria. It was also found that active films containing lignocellulose nanofibers and cellulose nanofibers had less antimicrobial activity than active films without these nanofibers. The effect of active films and nanocomposite produced in food models to increase and maintain food quality during the storage period was evaluated. Microbial evaluation of coated red meat pieces also showed that the use of edible film coating containing essential oil significantly reduced the growth rate of microorganisms compared to the uncoated sample. Thus, the highest contamination and microbial count was observed in the uncoated sample and then chitosan coating during the storage time. In contrast, especially active films containing essential oils, was able to significantly control the growth of these microorganisms at refrigerator temperature. The evaluation of the sensory properties of the meat samples also showed that pure chitosan film alone or together with essential oil increased the shelf life of red meat for more than 20 days at the refrigerator while the uncoated samples only were consumable for 10 days storage at the refrigerator.

Declarations

All authors declare that they have no conflict of interest.

Author contribution

Eisa Jahed: Investigation; Methodology & Formal analysis. Mohammad Alizadeh Khaledabad: Supervision and final editing. Khalid Esmahil Aziz: Writing–review & editing. Hadi Almasi: Writing – original draft. Hamed Hassanzadeh: Data curation; Validation and statistical analysis

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Footnotes

1. Plate Count Agar
2. Salmonella-Shigella Aga
3. Violet Red Bile Agar
4. Eosin Methylene Blue Agar
5. Sabouraud Dextrose Agar

Figures



Figure 1

Image of meat samples coated with chitosan-based active edible film

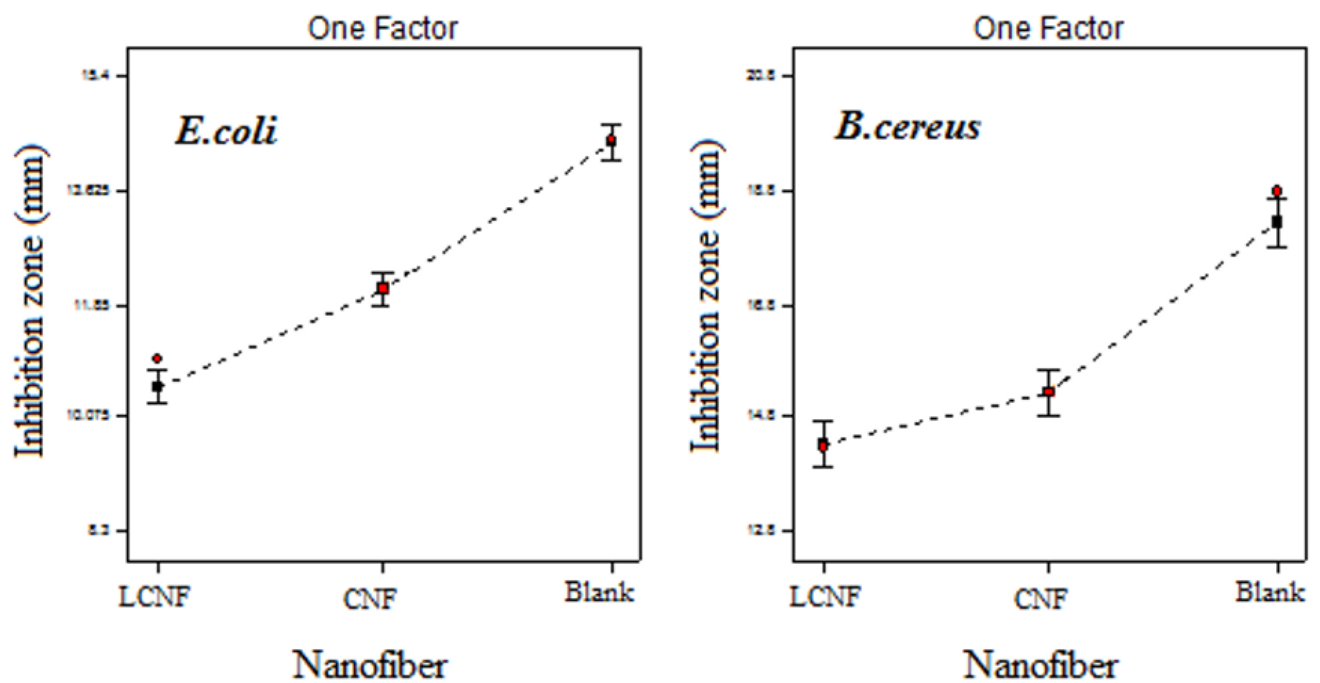


Figure 2

The effect of organic nanofibers on the size of the diameter of the non-growth halo of *E. coli* and *B. cereus* bacteria under the influence of chitosan-based active films

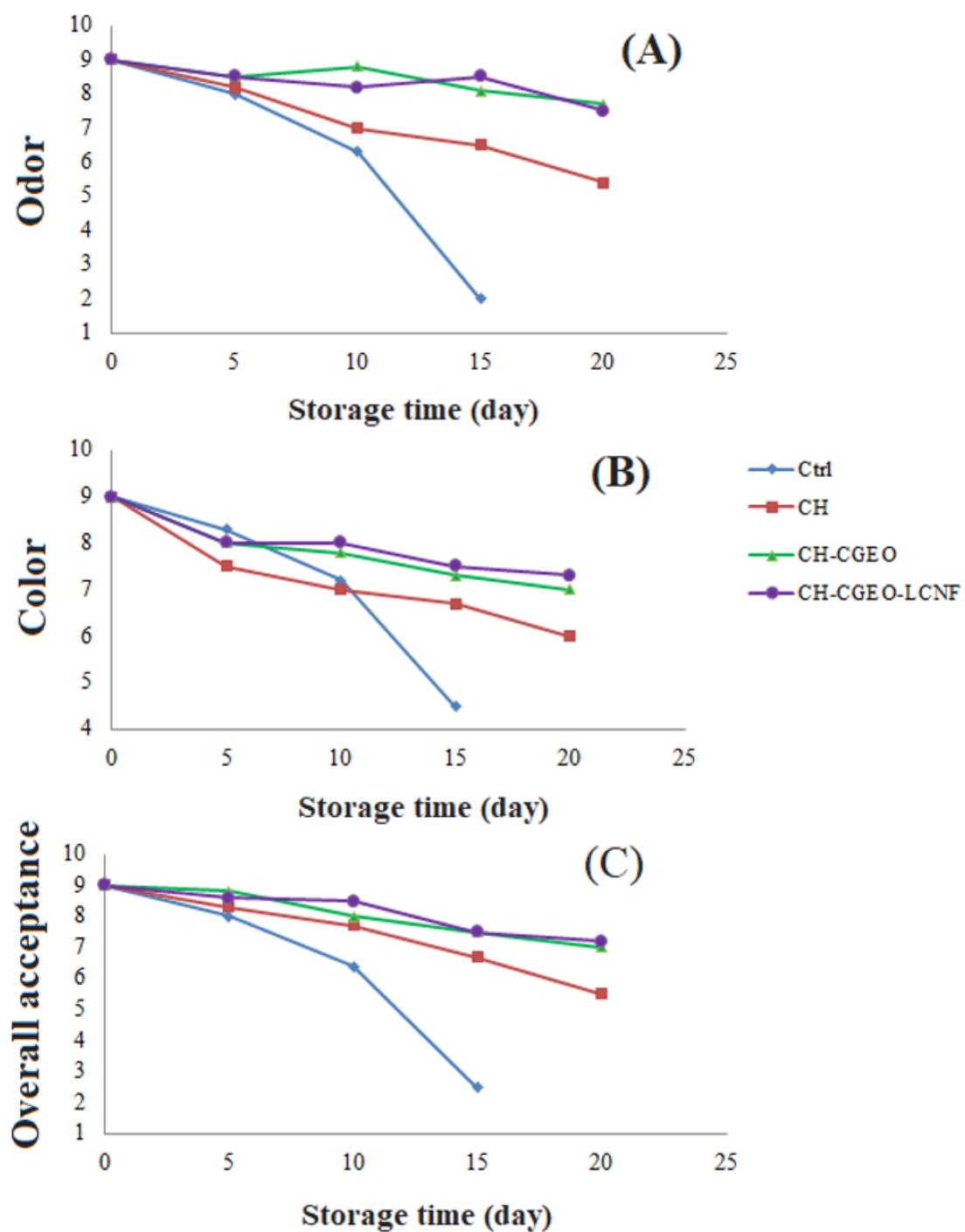


Figure 3

The effect of active chitosan films on the sensory characteristics of red meat stored for 20 days at 5°C. CH-CGEO= Active chitosan film with 5 % GEO/CEO and CH-CGEO-LCNF= Active chitosan film with combination of 5 % GEO/CEO and LCNF

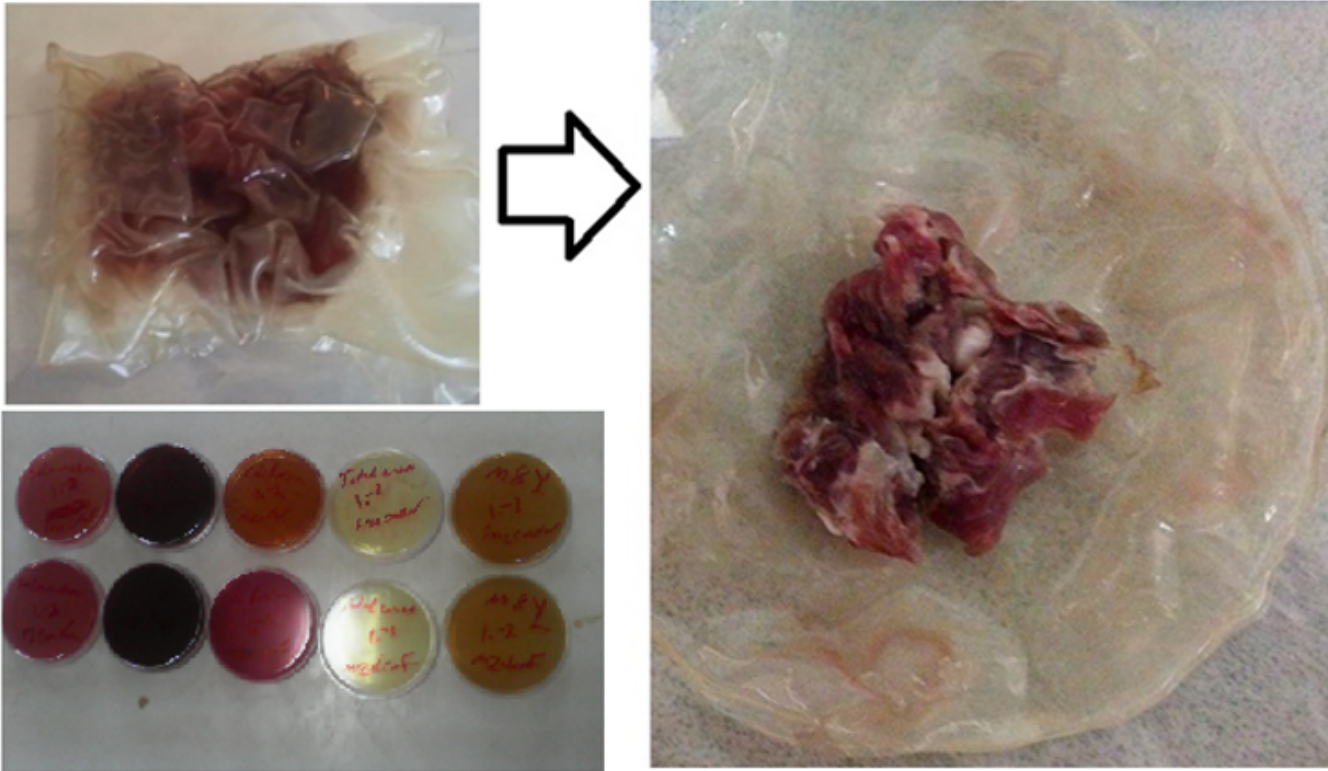


Figure 4

Image of meat samples coated with active chitosan film with combination of 5 % GEO/CEO and LCNF on the 20th day of storage

Supplementary Files

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