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Original article

Novel strategies of essential oils, chitosan, and nano- chitosan for inhibition of multi-drug resistant: *E. coli* O157:H7 and *Listeria monocytogenes*

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ABSTRACT

Despite the wide range of available antibiotics, food borne bacteria demonstrate a huge spectrum of resistance. The current study aims to use natural components such as essential oils (EOs), chitosan, and nano-chitosan that have very influential antibacterial properties with novel technologies like chitosan solution/film loaded with EOs against multi-drug resistant bacteria. Two strains of *Escherichia coli* O157:H7 and three strains of *Listeria monocytogenes* were used to estimate antibiotics resistance. Ten EOs and their mixture, chitosan, nano-chitosan, chitosan plus EO solutions, and biodegradable chitosan film enriched with EOs were tested as antibacterial agents against pathogenic bacterial strains. Results showed that *E. coli* O157:H7 51,659 and *L. monocytogenes* 19,116 relatively exhibited considerable resistance to more than one single antibiotic. Turmeric, cumin, pepper black, and marjoram did not show any inhibition zone against *L. monocytogenes*; Whereas, clove, thyme, cinnamon, and garlic EOs exhibited high antibacterial activity against *L. monocytogenes* with minimum inhibitory concentration (MIC) of 250–400 µl 100^{-1} ml and against *E. coli* O157:H7 with an MIC of 350–500 µl 100^{-1} ml, respectively. Among combinations, clove, and thyme EOs showed the highest antibacterial activity against *E. coli* O157:H7 with MIC of 170 µl 100^{-1} ml, and the combination of cinnamon and clove EOs showed the strongest antibacterial activity against *L. monocytogenes* with an MIC of 120 µl 100^{-1} ml. Both chitosan and nano-chitosan showed a promising potential as an antibacterial agent against pathogenic bacteria as their MICs were relatively lower against *L. monocytogenes* than for *E. coli* O157:H7. Chitosan combined with each of cinnamon, clove, and thyme oil have a more effective antibacterial activity against *L. monocytogenes* and *E. coli* O157:H7 than the mixture of oils alone. Furthermore, the use of either chitosan solution or biodegradable chitosan film loaded with a combination of clove and thyme EOs had the strongest antibacterial activity against *L. monocytogenes* and *E. coli* O157:H7. However, chitosan film without EOs did not exhibit an inhibition zone against the tested bacterial strains.

1. Introduction

Food safety is a critical issue in maintaining high-quality human food, and this issue is now a serious worry for a growing number of countries. Therefore, the food industry aims to produce high-quality and safe food stuffs (Panea and Ripoll, 2020; Saad et al., 2015, 2021a,b). Approximately 67% of foodborne diseases are caused by bacteria, 26% by chemicals, and approximately 4% by each of viruses and parasites (Addis and Sisay, 2015). The bacterial genera responsible for health hazards are *Bacillus, Campylobacter, Clostridium, Escherichia, Listeria, Salmonella, Shigella, Staphylococcus, Vibrio,* and *Yersinia* (Abd El-Hack et al., 2020; Bintsis, 2017).

Listeria monocytogenes causes serious diseases, such as listeriosis and bacteremia, as well as fatal diseases such as meningoencephalitis (Lecuit, 2020). *L. monocytogenes* is a major foodborne pathogen and causes issues in manufacturing plants (Thomas

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et al., 2020). The World Health Organization has listed *E. coli* among the 12 bacterial families that present the highest danger to human health. Notably, *E. coli* resistance to antibiotic treatment has been continuously growing (Serwecińska et al., 2021).

The resistance of bacteria to antibiotics is constantly increasing. Excessive and/or improper use of antibiotics will enhance this resistance. Consumer tendency to avoid foods containing chemicals with possible detrimental effect on health has led to the use of various natural substances (El-Saadony et al., 2021a,b), Generally Recognized as Safe (GRAS) (Bondi et al., 2017).

Over many decades, essential oils (EOs) have been used as antimicrobials, fungicides, antiparasitic agents, and virucides, in addition to their use in the fields of medicine and cosmetics (Butnariu and Sarac, 2015; El-Tarabily et al., 2021). Incorporating two or more natural EOs to exploit their antimicrobial properties greatly relies on both the composition and concentration of each oil (Abd El-Hack et al., 2021a; Cho et al., 2020).

Chitosan is a useful biomaterial for food preservation owing to its natural origin and superior biological qualities (Inanli et al., 2020). Nano-chitosan is a natural bioactive material against pathogenic bacteria such as *Staphylococcus aureus* and *Listeria* monocytogenes (Rozman et al., 2019a,b). Edible films and coatings made from chitosan have good potential for use in the preservation of food products, in addition to their use as EOs carriers. Compared with pure films and coatings, combining EOs with chitosan films increases both the antimicrobial effectiveness and antioxidant activity as well as their efficacy against postharvest fungi and foodborne bacteria in food products (Yuan et al., 2016).

This *in vitro* study was performed to evaluate the antibacterial activity of various natural agents such as EOs and their mixtures, solution of chitosan and nano-chitosan, solution/ biodegradable chitosan film loaded with EOs for use as antibacterial agents against multi-drug-resistant *L. monocytogenes* and *E. coli* O157:H7.

2. Materials and methods

2.1. Pathogenic bacterial strains

Five pathogenic bacterial strains, including *E. coli* O157:H7 ATCC 51659 and *L. monocytogenes* ATCC 19116 were purchased from the Microbiological Resource Center (MERCIN) at Faculty of Agriculture, Ain Shams University, Cairo, Egypt. *E. coli* O157: H7ATCC 6933, *L. monocytogenes* ATCC 19118, and *L. monocytogenes* ATCC 7644 were purchased from the Microbiological Laboratory of Animal Health Institute, Cairo, Egypt. The test bacteria were cultured on Mueller Hinton agar (MHA) (Jabbari et al., 2010) and then in tryptic soy broth (TSB) (Roberts et al., 1995) at 37 °C for 24 h and kept at 4 °C for further experiments. A loopful of each tested pathogenic bacteria (10^6 CFU/ml) determined by plate count assay was inoculated into a flask (100 ml) containing 50 ml of tryptic soy broth and incubated in a shaker incubator 150 rpm at 37 °C for 24 h.

2.2. Antibiotics

Twenty common antibiotics used in medical practice belonging to different groups were purchased from Oxoid, UK., and are shown in Table 1 (Aween et al., 2014). One milliliter of each bacterial inoculum (10^6 CFU/ml) was streaked on sterile Petri dishes containing MHA. The 20 antibiotic (Table 1) disks were placed on the center of inoculated plates and incubated at 37 °C for 24 h (Bauer et al., 1966). The results of sensitivity analysis of the tested bacteria to different antibiotics were categorized as sensitive, intermediate, and resistant according to Clinical Laboratory Standard Committee (CLSI., 2015).

2.3. Antibacterial activity of some chemical preservatives using disk diffusion method

Different concentrations of preservatives were prepared by dissolving them in Mueller Hinton Agar (MHA) (Jabbari et al., 2010). These preservatives solutions were heat-treated at 80 °C for 15 min before testing. The final concentrations of sodium benzoate and sodium nitrite were 1.0, 1.25, and 1.5 mg/ml and 1.0, 1.5, and 2.0 mg/ml, respectively. Whereas tri-sodium phosphate and sodium lactate at the same concentrations were 1%, 2%, and 3%. The multi-drug resistant pathogenic bacteria *E. coli* O157:H7 and *L. monocytogenes* were inoculated individually in Petri dishes containing tryptic soy agar medium (Roberts et al., 1995). Then, preservative impregnated discs were placed in the plates, and the plates were incubated for 24 h at 37 °C, according to the method reported previously (Stanojević et al., 2010).

2.4. Essential oils

The following 10 EOs (98% purity) were procured from the Medicinal and Aromatic Oils Unit at the National Research Center: thyme oil (*Thymus vulgaris*), turmeric oil (*Curcuma longa)*, parsley oil (*Petroselinum crispum*), garlic oil *(Allium sativum)*, cumin oil (*Cuminum cyminum*), clove oil (*Syzygium aromaticum*), pepper black oil (*Piper nigrum*), ginger oil (*Zingiber officinale*), cinnamon oil (*Cinnamomum zeylanicum*), and marjoram (*Origanum majorana*).

2.4.1. Antibacterial activity of EOs using agar well diffusion assay

One milliliter of *E. coli* O157:H7 6933 and *L. monocytogenes* 19,116 inoculum was spread onto sterile MHA. Using a sterile cork-borer, the 9-mm diameter well was cut from the agar, and subsequently, each well was filled with 100 µl of Eos either individual oil or their combinations (v/v). The plates were incubated for 1 h at room temperature and then for 24 h at 37 °C according to the method described previously (López et al., 2005). Commercially available gentamicin disk (30 µg) was used as a positive control. The inhibition zone was determined in millimeters.

2.4.2. Minimum inhibitory concentration (MIC) for EOs

The four most effective EOs, i.e., cinnamon, clove, thyme, and garlic EOs, against *L. monocytogenes* and *E. coli* O157:H7 were selected based on their antimicrobial activity. Briefly, 500 µl of tested bacterial strains (10^6 CFU/ml) were inoculated in 4.0 ml of Mueller Hinton Broth (MHB) (Jabbari et al., 2010) and mixed with 50–500 µl/100 ml of each EO supplemented with tween 80% (0.01% v/v) and then incubated at 37 °C for 24 h. MIC was defined as the lowest concentration that completely inhibited the visible growth of bacteria in broth medium and was confirmed by re-inoculating on MHA (Berche et al., 1996).

2.4.3. Determination of the MICs of EO combinations

To determine the MIC of EO combinations, broth macro dilution assays were performed (CLSI., 2017). Briefly, 500 µl of each tested bacterium was inoculated in MHB tubes by mixing with 50–500 µl/100 ml of each combination of EOs (El-Saadony et al., 2021c).

2.4.4. Synergistic effect

The synergistic effect of EO combinations was estimated by determining the fractional inhibition concentration (FIC) index for each combination. FIC was calculated using the following equations (Davidson and Parish, 1989):

$$1 - \mathrm{FIC1} = \frac{\mathrm{MIC\ of\ A/B}}{\mathrm{MIC\ of\ a}}$$

Table 1
Sensitivity of *E. coli* O157:H7 to different antibiotics.

Antibiotics	Disk content (μg/ml)	*E. coli* O157:H7 (ATCC 1659)	*E. coli* O157:H7 (ATCC 6933)		
		Inhibition zone (mm)	Interpretive standard of (I.Z)	Inhibition zone (mm)	Interpretive standard of (I.Z)
Penicillin	10	11.3	R	10.0	R
Ampicillin	10	15.5	I	17.8	S
Amoxicillin + clavulanic acid	30	12.5	R	15.5	I
Cephalexin g1	30	11.5	R	12.8	R
Ceftriaxone g3	30	12.7	R	14.5	R
Cefaclor g2	30	N.I	R	10.5	R
Ceftazidime g3	30	N.I	R	9.5	R
Rifampicin	5	16.25	I	19.5	S
Vancomycin	30	9.0	R	17.5	S
Azithromycin	15	10.5	R	13.0	S
Amikacin	10	15.5	I	13.5	I
Gentamicin	10	15.5	S	17.0	S
Oxytetra acid	10	10.5	R	22.0	S
Doxycycline	30	10.7	I	13.0	I
Colistin	10	8.0	S	16.0	S
Sulfamethoxazole	30	13.5	I	15.0	I
Cidocetine	30	10.2	R	10.7	R
Ciprofloxacin	5	12.0	R	22.0	S
Levofloxacin	5	N.I	R	8.50	R
Nitrofurantoin	30	4.5	R	6.5	R

R, Resistant; I, Intermediate; S, Sensitive; CLSI, Clinical Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; BSAC, Britch Society for Antimicrobial chemotherapy; N.I, No Inhibition.

$$2 - \text{FIC2} = \frac{\text{MIC of A/B}}{\text{MIC of b}}$$

FIC = FIC_1 + FIC_2, A/B = combination oil, a/b = individual oil
FIC index < 1: synergistic effect, = 1: additive effect, > 1: antagonistic effect.

2.5. Chitosan and nano-chitosan characterization

Chitosan powder (molecular weight: 100,000–300,000; degree of deacetylation: 75%, white powder, spherical, odorless, completely stable, and non-toxic) was obtained from ACROS ORGANICS (Belgium). While nano-chitosan (size: 50–100 nm) was purchased from Nano-Fab Technology, New Maadi, Cairo.

2.5.1. Antibacterial activity of chitosan and nano-chitosan using agar well diffusion assay

Briefly, 9-mm wells were punched over the agar plates. Chitosan (2 g) and nano-chitosan (2 mg) were dissolved in distilled water and acetic–glacial acid mixture (100:1 v/v), respectively, to obtain their solutions. Subsequently, chitosan and nano-chitosan solutions of 25, 50, 75, and 100 μl/plate were placed in the wells. These plates were kept at room temperature for 1 h and then incubated at 37 °C for 24 h. At the end of the incubated period, the diameter of the inhibition zone was measured (Aliasghari et al., 2016).

2.5.2. Determination of MIC for chitosan and nano-chitosan

One milliliter of each bacterial inoculum was individually added to tubes containing MHB medium with chitosan in serial two-fold dilution (1, 2, 4, 8, 16, 32, 64, 128, 156, and 512 μg /ml) and with nano-chitosan in serial two-fold dilution (0.8, 1.6, 3.2, 6.4, 12.8, 25.6, 51.2, and 102.4 μg/ml). The control tube was free from chitosan and nano-chitosan. These tubes were then incubated at 37 °C for 24 h (El-Saadony et al., 2021d).

2.6. Preparation of chitosan and nano-chitosan combined with EOs

The MIC of either chitosan or nano-chitosan was mixed with the MIC of each cinnamon, thyme, clove, and garlic EO as well as with cinnamon + clove EO and thyme + clove EO and was supplemented with 0.01% of tween 80% with constant stirring at room temperature for 4–6 h. Fresh chitosan or nano-chitosan solutions loaded with various EOs were used as antibacterial agents against pathogenic bacteria (Chi et al., 2006).

2.7. Preparation of EO-loaded chitosan films

The chitosan films were prepared by dissolving chitosan in an aqueous (1% w/v) solution with glacial acetic acid (1% w/v) and then stirring on a magnetic stirrer hot plate at 50 °C. The MICs of cinnamon, clove, and thyme EOs; cinnamon + clove EO; and clove + thyme EO were added to chitosan solution, followed by stirring from 3 to 6 h. Glycerol 30% was mixed with chitosan–oil mixture in the beaker along with tween 80% at 0.2% (v/v); this solution was homogenized at 4000 rpm for 6 h to ensure emulsion formation. The mixtures were poured into a plastic Petri dish to dry at room temperature for at least 72 h. After drying, the membrane could be removed easily (Mehdizadeh et al., 2012).

2.7.1. Determination of antibacterial effect EO-loaded chitosan films by direct contact

Discs (12 mm) were cut from the films and placed on MHA plates inoculated with 0.1 ml of bacterial inoculum at 10^6 CFU/ml. These plates were then incubated at 37 °C for 24 h, and then the inhibition zone was measured (Seydim and Sarikus, 2007).

2.7.2. Determination of the antioxidant activity of EO-loaded chitosan film

The antioxidant activities in EO-loaded chitosan were determined following (Saad et al., 2021c) by measuring the alterations in the DPPH· purple-colored solution. An aliquot of 100 μl of each sample was added to 1 ml methanolic DPPH· and kept for 30 min at room temperature before measuring the absorbance (A) at

517 nm against the purple color. The DPPH scavenging activity (%) was calculated according to the following equation:

$$\text{DPPH scavenging effect}(\%) = \frac{\text{AbsDPPH} - \text{AbsExtract}}{\text{AbsDPPH}} \times 100$$

where Abs_{DPPH} is the absorbance value at 515 nm of the methanolic solution of DPPH, and $Abs_{extract}$ is the absorbance value at 515 nm of sample extracts (Ashry et al., 2022).

2.7.3. Total phenols

Total phenols were determined according to the method of (Elhakem et al., 2020; Saad et al., 2021d).

3. Results

3.1. Sensitivity of pathogenic bacterial strains to different antibiotics

As shown in Table 1, *E. coli* O157:H7 ATCC 51659 had a higher resistance than *E. coli* O157:H7ATCC 6933 by (65% and 40%, respectively) of the tested antibiotics. Based on the obtained results, the two strains of *E. coli* O157:H7 can be classified as multi-drug resistant bacteria.

3.2. Sensitivity of L. monocytogenes to different antibiotics

As shown in Table 2, *L. monocytogenes* ATCC 19116 was more resistant by 60%, *L. monocytogenes* ATCC 7644 showed a high resistance by 55% while *L. monocytogenes* ATCC 19118 was resistant to a low by 25% of all tests antibiotics.

3.3. Antibacterial activity of preservatives

As shown in Table 3, the inhibition area increased with increasing concentration of sodium benzoate, sodium nitrite, and sodium tripolyphosphate.

Sodium nitrite had the maximum inhibition zone of 16 mm against *L. monocytogenes* at a concentration of 2.0 mg/ml. Sodium lactate showed a higher inhibition zone for *L. monocytogenes* than for *E. coli* O157:H7 (16.0 and12.0 mm, respectively). While, the inhibition zone of sodium tripolyphosphate against *L. monocytogenes* and *E. coli* O157:H7 (14.8 and 11.6 mm, respectively) was the lowest compared to other preservatives.

Table 3
Inhibition zone of concentrated sodium benzoate, sodium nitrite, sodium tripolyphosphate, and sodium lactate against pathogenic bacteria.

Preservatives	Bacterial strains	
	E. coli O157:H7 (ATCC 6933)	*Listeria monocytogenes* (ATCC 19116)
Inhibition zone (mm)		
Disc saturated with sterile water as a control	N.I	N.I
Sodium benzoate (mg/ml)		
1.00	6.5	13.2
1.25	8.3	14.6
1.50	12.3	16.0
Sodium nitrite (mg/ml)		
1.0	10.0	14.0
1.5	13.0	16.0
2.0	15.0	17.5
Sodium tripolyphosphate (%)		
1.0	9.5	11.0
2.0	10.5	12.4
3.0	11.6	14.8
Sodium lactate (%)		
1.0	9.6	11.5
2.0	11.0	14.0
3.0	12.0	16.0

3.4. Antibacterial activity of tested EOs

As shown in Table 4, most EOs inhibited the growth of the tested bacterial strains, and the inhibition zone varied depending on EOs selected and the bacterial strains. Cinnamon, clove, thyme, and garlic EOs had the highest antibacterial activity against *L. monocytogenes* and *E. coli* O157: H7. In contrast, EOs of turmeric, cumin, black pepper, and marjoram had a small inhibition zone against *L. monocytogenes* and showed no antibacterial activity against *E. coli* O157:H7. Additionally, *L. monocytogenes* showed more sensitivity to EOs than *E. coli* O157:H7.

Table 2
Sensitivity of *Listeria monocytogenes* to different antibiotics.

Antibiotics	Disk content (μg/ml)	*Listeria monocytogenes* (ATCC 7644)		*Listeria monocytogenes* (ATCC 19116)		*Listeria monocytogenes* (ATCC 19118)	
		Inhibition zone (mm)	Interpretive standard of I.Z	Inhibition zone (mm)	Interpretive standard of I.Z	Inhibition zone (mm)	Interpretive standard of I.Z
Penicillin	10	10.5	R	12.0	R	36.0	S
Ampicillin	10	10.5	R	12.0	R	21.0	S
Amoxicillin + Clavulanic acid	30	9.5	R	11.0	R	18.5	S
Cephalexin g1	30	15	R	14.2	R	15.5	R
Ceftriaxone g3	30	12.5	I	11.8	I	18.7	S
Cefaclor g2	30	7.5	R	5.0	R	11.5	R
Ceftazidime g3	30	N.I	R	N.I	R	11.0	R
Rifampicin	5	14.5	R	12.5	R	18.5	I
Vancomycin	30	8	R	7.0	R	20.5	S
Azithromycin	15	18	S	16.0	I	14.5	I
Amikacin	10	17.0	S	15.0	S	17.0	S
Gentamicin	10	15.5	S	16.0	S	23.0	S
Doxycycline	30	11.6	R	12.5	R	20.6	S
Oxytetra acid	10	14.5	I	13.0	R	18.5	I
Colistin	10	8	S	12.0	I	12.5	I
Sulfamethoxazole	30	31	S	28.0	S	34.0	S
Ciprofloxacin	5	20	I	18.7	I	21.5	S
Levofloxacin	5	10.0	R	N.I	R	10.0	R
Cidocetine	30	15.5	I	14.8	I	16.4	S
Nitrofurantoin	30	11.5	R	N.I	R	10.5	R

R, Resistant; I, Intermediate; S, Sensitive; CLSI, Clinical Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; BSAC, Britch Society for Antimicrobial chemotherapy; N.I, No Inhibition.

Table 4
Antibacterial activity of essential oils against pathogenic bacteria.

Essential oils	Bacterial strains	
	E. coli O157:H7 (ATCC6933)	*Listeria monocytogenes* (ATCC 19116)
Inhibition zone (mm)		
Thyme	15.0	24.5
Turmeric	N.I	10.0
Parsley	11.8	16.8
Garlic	16.0	21.5
Cumin	N.I	13.0
Clove	21.5	26.0
Pepper black	N.I	10.0
Ginger	10.0	13.5
Cinnamon	22.0	26.8
Marjoram	N.I	10.5
Gentamycin (30 µg/mL)	15.0	16.5

N.I, No Inhibition (<9 mm diameter).

3.5. MIC of EOs against pathogenic bacteria

As shown in Table 5, MIC of clove and cinnamon at (350 µl/100 ml) against *L. monocytogenes* and at (250 µl/100 ml) against *E. coli* O157:H7. Garlic oil had high MIC at (500 and 400 µl/100 ml) against *E. coli* O157:H7 and *L. monocytogenes*, respectively compared to the MIC of other oils.

3.6. Effect of combinations of EOs

As shown in Table 6, the combination of cinnamon and clove EOs showed had the strongest antibacterial activity against *L. monocytogenes* with an inhibition zone of 35.0 mm. In contrast, the highest inhibition zone of 31.5 mm was recorded against *E. coli* O157:H7 for a combination of clove and thyme EOs. These results are in harmony with those reported previously (Purkait et al., 2018). The combination of thyme and garlic EOs showed the lowest inhibition zone of 28.4 and 21.0 mm against *L. monocytogenes* and *E. coli* O157:H7, respectively compared to other mixtures of oil. Based on the FIC index, shown in Table 6, all combinations showed a synergistic effect against two bacterial strains, except thyme + garlic EO combination, which exhibited an additive effect against both the selected bacterial strains, and clove + garlic EO, which showed an additive effect against only *E. coli* O157:H7.

The combination of cinnamon and clove EOs (1:1, v/v) exhibited a clear synergistic effect against *L. monocytogenes* and *E. coli* O157:H7, as the MICs were 120 and 170 µl/100 ml, respectively. In contrast, thyme + garlic EOs demonstrated an additive effect against *L. monocytogenes* and *E. coli* O157:H7, as the MICs were 180 and 240 µl/100 ml, respectively.

3.7. Antibacterial activity of chitosan and nano-chitosan

As shown in Table 7, chitosan and nano-chitosan markedly inhibited the growth of tested bacterial strains. However, different inhibition zones were recorded for different solution used (25, 50, 75, and 100 µl/plate) at a 2% concentration and the type of pathogenic bacteria. Chitosan at 100 µl/plate showed antibacterial activity against *L. monocytogenes* ATCC 19116 and *E. coli* O157:H7 ATCC 6933, with a wide inhibition zone of 28.6 and 25.0 mm, respectively. Nano-chitosan at 100 µl/plate showed a higher inhibition zone against *L. monocytogenes* than against *E. coli* O157:H7.

Although chitosan at 2% concentration exhibited an antimicrobial effect against the tested bacterial strains, nano-chitosan showed a higher inhibition zone than chitosan at the same concentration.

Chitosan had a higher MIC against *E. coli* O157:H7 ATCC6933 than against *L. monocytogenes* ATCC 19116 (256 and 64 µg/ml, respectively). *L. monocytogenes* was more sensitive to nano-chitosan with a lower MIC than to *E. coli* O157:H7.

3.8. Effect of chitosan and nano-chitosan combined with EOs against pathogenic bacteria

As shown in Table 8, the lowest inhibition zone against *E. coli* O157:H7 ATCC6933 and against *L. monocytogenes* ATCC 19116 was observed for chitosan enriched with garlic EO compared to chitosan mixed with other oils. The combination of chitosan and thyme EO had a higher inhibition zone against *L. monocytogenes* than against *E. coli* O157:H7. The combination of chitosan with clove EO showed stronger antibacterial activity than chitosan with cinnamon EO against *L. monocytogenes* and *E. coli* O157:H7 (Table 9). These results are in line with those reported previously (Mukhtar et al., 2018).

Nano-chitosan combined with EOs showed a lower inhibition zone than chitosan combined with the same oils against *L. monocytogenes* and *E. coli* O157:H7. The mixture of clove + thyme EO combined with chitosan had the highest inhibition zone of 42.5 and 35.0 mm against *L. monocytogenes* and *E. coli* O157:H7, respectively.

3.9. Total phenolic content and antioxidant activity against DPPH in biodegradable chitosan film

Total phenolic content of each chitosan film enriched with thyme, cinnamon, clove, cinnamon + clove, and clove + thyme EOs was 6.52, 5.43, 5.50, 7.34, and 8.01 mg/ml, respectively in Table 10. The highest total phenolic content was observed for clove + thyme EO compared to with other oils.

The addition of EOs onto chitosan films enhanced their antioxidant properties compared with the control films, and this enhancement was depending on the type of EO used.

Chitosan film without EO (control) showed a low scavenging activity on DPPH, whereas chitosan film enriched with EOs had greater values. The highest value of DPPH (93%) was obtained with chitosan film + clove + thyme, and chitosan film + cinnamon EO had the lowest one.

3.10. Biodegradable chitosan film loaded with EOs

As shown in Table 10, chitosan film combined with clove EO had greater antibacterial activity against *Listeria monocytogenes* ATCC 19116 and *E. coli* O157:H7 ATCC 6933 than chitosan film

Table 5
Minimal inhibition concentration (MIC) of essential oils against pathogenic bacteria.

Bacterial strains	Values of MIC for essential oils (µl/100 ml)			
	Clove	Thyme	Cinnamon	Garlic
E. coli O157:H7 (ATCC6933)	350	400	350	500
Listeria monocytogenes (ATCC 19116)	250	350	250	400

Table 6
Effect of essential oil combinations against pathogenic bacteria.

Bacterial strains	Essential oil mixtures	Inhibition zone of different essential oil mixtures (mm)	MIC of essential oils mixtures (µl/100 ml)	FIC (index)	Effect of combination
E. coli O157:H7 (ATCC6933)	Cinnamon + Clove	29.5	180	0.96	Synergistic
	Cinnamon + Garlic	28.3	200	0.97	Synergistic
	Cinnamon + Thyme	28.8	170	0.90	Synergistic
	Clove + Thyme	31.5	170	0.96	Synergistic
	Clove + Garlic	26.5	230	1.0	Additive
	Thyme + Garlic	21.0	240	1.0	Additive
L. monocytogenes (ATCC 19116)	Cinnamon + Clove	35.0	120	0.96	Synergistic
	Cinnamon + Garlic	31.6	140	0.91	Synergistic
	Cinnamon + Thyme	32.5	120	0.82	Synergistic
	Clove + Thyme	32.0	140	0.96	Synergistic
	Clove + Garlic	30.0	150	0.97	Synergistic
	Thyme + Garlic	28.4	180	0.96	Additive

Table 7
Antibacterial activity of chitosan and nano-chitosan pathogenic bacteria.

Antibacterial agents	Bacterial strains	
	E. coli O157:H7 (ATCC 6933)	*L. monocytogenes* (ATCC 19116)
Chitosan/plate (µl/ml)	Inhibition zone (mm)	
25	14.0	22.0
50	15.0	23.8
75	23.0	28.5
100	25.0	28.6
MIC (µg/ml)	256	64
Nano-chitosan/plate (µl/ml)	Inhibition zone (mm)	
25	19.0	25.0
50	21.6	28.5
75	24.8	30.0
100	28.5	30.0
MIC (µg/ml)	51.2	12.8

Table 8
Chitosan and nano-chitosan combined with essential oils against pathogenic bacteria.

Antibacterial agents (µl/ml)	Bacterial strains	
	E. coli O157:H7 (ATCC 6933)	*L. monocytogenes* (ATCC 19116)
Inhibition zone (mm)		
Chitosan + garlic	27.6	30.0
Chitosan + thyme	32.0	38.0
Chitosan + cinnamon	28.0	32.6
Chitosan + clove	30.6	36.0
Chitosan + (cinnamon + clove)	33.5	40.0
Chitosan + (clove + thyme)	35.0	42.5
Nano-chitosan + garlic	17.0	23.0
Nano-chitosan + thyme	15.0	24.0
Nano-chitosan + cinnamon	12.0	23.0
Nano-chitosan + clove	19.0	28.0
Nano-chitosan + (cinnamon + clove)	21.0	27.3
Nano-chitosan + (clove + thyme)	25.0	30.0

combined with cinnamon EO. Chitosan film incorporated with thyme oil had a stronger antibacterial activity against *L. monocytogenes* than against *E. coli* O157:H7. This result is in agreement with that reported previously (Jovanovic et al., 2016). It is important to mention that compared with chitosan films without EOs, those enriched with EOs showed higher antibacterial activities against all tested bacterial strains. Chitosan film enriched with a combination of clove + thyme EO showed the highest inhibition zone of 41.5 and 33.0 mm against *L. monocytogenes* and *E. coli* O157:H7, respectively.

Table 9
Total phenolic content and antioxidant activity against DPPH of chitosan film incorporated with essential oils against pathogenic bacteria.

Antibacterial agents	Total phenol content (mg/ml)	Antioxidant activity DPPH (%)
Chitosan film (control)	0.00	42.3
Chitosan film + thyme	6.52	74.0
Chitosan film + cinnamon	5.43	71.7
Chitosan film + clove	5. 50	79.6
Chitosan film + (cinnamon + clove)	7. 34	89.8
Chitosan film + (clove + thyme)	8.01	93.0

4. Discussion

Multidrug resistance among bacteria is now one of the most pressing issues in global public health. The two strains of *E. coli* O157:H7 can be classified as multi-drug resistant bacteria, according to a previous study (Xu et al., 2020). This may be attributed to the lipopolysaccharides in the cell wall of *E. coli* O157:H7; these act as a strong barrier toward antibiotics, causing bacteria to be resistant several to theirs. In addition members of Enterobacteriaceae can produce β-lactamases that can allow these bacteria to be resistant to β-lactam antibiotics by hydrolyzing the β-lactam ring in the antibiotics (Miller, 2016).

Additionally, all *L. monocytogenes* strains were multi-drug resistant. These results are in agreement with those reported in a previous study (Abdeen et al., 2021). The multi-drug resistance of *L. monocytogenes* strains could be attributed to two types of resistance demonstrated by the bacteria: innate and acquired resistance. *Listeria* spp. exhibit an innate resistance to a variety of antimicrobials including many β-lactams, most of the cephalosporins (Krawczyk-Balska and Markiewicz, 2016).

The inhibition area increased with increasing preservatives concentration, this was in line with that reported previously (El-Saadony et al., 2022; Saranraj, 2012). Sodium nitrite gave maximum inhibition zone, similar to that demonstrated previously (Majou and Christieans, 2018). Nitrite salts are effective antimicrobial agents, probably due to their effects that involve decreasing water potential, delaying oxidative rancidity, and subsequently preventing the growth of bacteria (Crowe et al., 2020). The effectiveness of sodium lactate against *E. coli* O157:H7 and *L. monocytogenes* might be explained by its binding to acid, crossing the

Table 10
Chitosan film (CF) loaded with essential oils against pathogenic bacteria.

Antibacterial agents	CF (control)	CF + thyme	CF + cinnamon	CF + clove	CF + cinnamon + clove	CF + clove + thyme
	Inhibition zone (mm)					
E. coli O157:H7 ATCC 933	N.D	32.0	25.0	30.0	32.5	33.0
L. monocytogenes ATCC 19,116	N.D	36.0	31.0	33.0	39.0	41.5

microbial cell membrane, and increasing the acidity of cell interior (Carpenter and Broadbent, 2009). While, ssodium tripolyphosphate gave the lowest inhibition zone and these results are in similar to those reported previously (Jang et al., 2016), however, the natural antibacterial agents were more effective and safe (Abd El-Hack et al., 2021b; Alagawany et al., 2021a).

Novel and more effective antibacterials are needed to address this challenge, most EOs like cinnamon, clove, thyme, lemongrass and garlic inhibited the growth of the tested bacterial strains as discussed by Alagawany et al. (2021b). In contrast, EOs of turmeric, cumin, black pepper, and marjoram had a small inhibition zone, these results are in agreement with those reported previously (Franco, 2007). The antimicrobial activity of EOs may be attributed to their bioactive volatile components (Yousefi et al., 2020). Additionally, *L. monocytogenes* showed more sensitivity to EOs than *E. coli* O157:H7. This can be explained by several mechanisms including the more resistant nature of Gram-negative bacteria owing to the double layer of phospholipids in their cell membrane (Bhavaniramya et al., 2019).

The antibacterial activity of these oils has been largely attributed to the presence of cinnamaldehyde and eugenol (Abdelwahab et al., 2014). Minimum inhibitory concentrations (MIC) are defined as the lowest concentration of an antimicrobial which inhibit the visible growth of a microorganism after overnight incubation (Chi et al., 2006; Desoky et al., 2020). Garlic oil had high MIC the tested bacterial compared to the MIC of other oils. This result was in agreement with that reported previously (Kim and Fung, 2004). In contrast, another study (Jolly and K, 2015) reported that garlic possesses a good potential against pathogenic bacteria.

The cinnamon and clove combination showed the strongest antibacterial activity against *L. monocytogenes* while, the highest inhibition zone was recorded against *E. coli* O157:H7 by clove and thyme combination. These results are in harmony with those reported previously (Purkait et al., 2018). The higher efficacy of oil combinations compared with individual oils might be attributed to either the inhibition of common biological pathways in microorganisms, suppression of protective enzymes, or modification of cell wall functions. EOs consist of different chemical compounds that may have different antimicrobial modes of action. Therefore, the possibility of antimicrobial resistance is minimized (Ambrosio et al., 2016).

Chitosan and nano-chitosan showed promising antimicrobial activity against several food born pathogenic bacteria. Nano-chitosan showed a higher inhibition zone against *L. monocytogenes* than against *E. coli* O157:H7. These data are in line with those reported previously (Abdeltwab et al., 2019). Although chitosan at 2% concentration exhibited an antimicrobial effect against the tested bacterial strains, nano-chitosan showed a higher inhibition zone than chitosan at the same concentration. This may be attributed to the features of nano-chitosan (Rozman et al., 2019a,b).

Chitosan had a higher MIC against *E. coli* O157:H7 ATCC6933 than against *L. monocytogenes* ATCC 19116. These results are in agreement with those reported previously (El-Dahma et al., 2017). *L. monocytogenes* was more sensitive to nano-chitosan with a lower MIC than to *E. coli* O157:H7. This result is in line with that reported previously (Ke et al., 2021).

The combination of chitosan and thyme EO had a higher inhibition zone against *L. monocytogenes* than against *E. coli* O157:H7. This result is in line with that reported previously (Raphaël and Meimandipour, 2017a,b). Additionally, the combination of chitosan with clove EO showed stronger antibacterial activity than chitosan with cinnamon EO against *L. monocytogenes* and *E. coli* O157:H7. These results are in line with those reported previously (Mukhtar et al., 2018). Nano-chitosan combined with EOs showed a lower inhibition zone than chitosan combined with the same oils against *L. monocytogenes* and *E. coli* O157:H7. These results may be attributed to when nano-chitosan was mixed with oils, its properties have changed. (Ramezani et al., 2015) showed that nano-chitosan exhibited higher antimicrobial activity than chitosan against foodborne pathogens.

The mixture of clove + thyme EO combined with chitosan had the highest inhibition zone against the tested bacteria According to (Pereira dos Santos et al., 2020) who reported that thyme and clove EOs were very active when combined with chitosan. This high antibacterial activity that was recorded against *E. coli* O157:H7could be explained by the fact that the positively charged chitosan enriched with EOs can create a semi-permeable barrier capable of reducing respiration and retarding growth (Raphaël and Meimandipour, 2017a,b).

The highest total phenolic content was observed for clove + thyme EO compared to with other oils. This result is in agreement with that reported previously (Alparslan, 2018). Chitosan film without EO (control) showed a low scavenging activity on DPPH, whereas chitosan film enriched with EOs had greater values. The highest value of DPPH (93%) was obtained with chitosan film + clove + thyme, and chitosan film + cinnamon EO had the lowest one. The antioxidant activity exhibited by chitosan films enriched with EOs could be attributed to bioactive compounds such as phenolic acids or terpenoids in EOs (Ruiz-Navajas et al., 2013).

A previous study (Venkatachalam and Lekjing, 2020) reported that chitosan incorporated with clove oil could enhance its antimicrobial properties. Also, chitosan film incorporated with thyme oil had a stronger antibacterial activity against *L. monocytogenes* than against *E. coli* O157:H7. This result is in agreement with that reported previously (Jovanovic et al., 2016). It is important to mention that compared with chitosan films without EOs, those enriched with EOs showed higher antibacterial activities against all tested bacterial strains. This phenomenon could be attributed to the fixing of chitosan molecules within the film matrix, which avoided their diffusion through the agar medium (Wang et al., 2011). Chitosan film enriched with a combination of clove + thyme EO showed the highest inhibition zone against *L. monocytogenes* and *E. coli* O157:H7. This may be related to phenolic compounds (Pei et al., 2009), which disrupt the cell membrane, increasing permeability. In addition, they could interact with membrane proteins, deforming the structure and functionality (Viuda-Martos et al., 2007).

Members of Enterobacteriaceae can produce β-lactamases that can allow these bacteria to be resistant to β-lactam antibiotics by hydrolyzing the β-lactam ring in the antibiotics (Miller, 2016).

5. Conclusion

Multidrug resistance among bacteria is now one of the most pressing issues in global public health. So, novel and more effective

antibacterials are needed to address this challenge. In view of the obtained results, it could be concluded that chitosan solution and biodegradable films loaded with EOs are more effective than utilizing oils, chitosan, and nano-chitosan separately as antibacterial activity against pathogenic bacteria. Therefore, the use of chitosan loaded with EOs could be recommended for food preservation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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