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The Potential Synergistic Activity of Chitosan-Essential Oils Combination for Fighting Multidrug-Resistant *Salmonella* Typhimurium and *Staphylococcus aureus*

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Multidrug resistance among bacteria is now one of the most pressing issues in global public health. So, novel and more effective antibacterial materials are needed to address this challenge. The current study aims to use natural antibacterial agents and new strategies to prevent the growth of multidrug-resistant *Salmonella* Typhimuṛium and *Staphylococcus aureus*. Ten essential oils (EOs) and their mixture, chemical preservatives, chitosan, nano-chitosan, chitosan solution/film loaded with Eos were tested as antibacterial agents against pathogenic bacterial strains. *S.* Typhimurium ATCC 25566 and *Staph. aureus* ATCC 6538 were the most resistant to several antibiotics. Each essential oil of turmeric, cumin, pepper black, and marjoram, had no effect on *S.* Typhimurium while *Staph. aureus* was sensitive to them. However, clove, thyme, cinnamon, and garlic EO showed the maximum effect on *S.* Typhimurium and *Staph. aureus*. Their minimal inhibition concentration (MIC) was (350, 400, 350, and 500 µl 100-1) against *S.* Typhimurium and (250, 350, 250, and 400 µl 100-1 ml) against *Staph. aureus*, respectively. The mixture of clove and thyme recorded higher antibacterial activity values against *S.* Typhimurium and *Staph. aureus* compared to the mixture of other oils. Chitosan and nano-chitosan demonstrated potent antibacterial activity against *S.* Typhimurium and *Staph aureus*. *Staph. aureus* was more sensitive to nano chitosan. The mixture solution of chitosan, clove, and thyme was the most active combination against *S.* Typhimurium and *Staph. aureus*. Biodegradable chitosan film loaded with EOs was more effective antibacterial activity against *S.* Typhimurium and *S. aureus* than chitosan-free-essential oils films.

Keywords: Antibiotic-resistance, *Salmonella* Typhimuṛium, *Staphylococcus aureus*, Essential oils, Chitosan, Nano-chitosan, Biodegradable film

Introduction

Foodborne pathogens are becoming increasingly resistant to antimicrobial compounds due to overuse and abuse. Bacteria resistant to antibiotics have been found in all stages of the food chain from farm to fork, highlighting the huge issue that is antimicrobial-resistant (Giacometti et al., 2021). *Listeria*, *Salmonella*, *Staphylococcus*, *Vibrio*, and *Yersinia* are the common pathogenic bacteria responsible for health risks and nowadays show high resistance among them (Bintsis, 2017). *Staphylococcus aureus* is responsible for many infections: bacteremia; endocarditis, and pulmonary system infections and this bacterium has nowadays exhibited great antibiotic resistance (Tong et al., 2015). *Salmonella* Typhimuṛium is the most important cause of foodborne illness and its recent skyrocket in antibiotic resistance over years has made it of very worrying concern since this likely leads to harsh health breaks (Tong et al., 2015).

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Despite that food additives are widely used to prevent food spoilage and inhibit pathogenic bacteria, there is still a controversy over their extensive use in food preservation and their detrimental effect on health (Awuchi et al., 2020). The current trends in the food processing industry throw the light on using natural compounds which are considered more safe alternatives (Bondi et al., 2017). Essential oils are considered as great potential bio-preservatives to minimize or eliminate pathogenic bacteria in processed food products (Zhang et al., 2017). Oils mixture may be considered a good candidate as a unique natural antimicrobial and antioxidant agent (Purkait et al., 2020).

Chitosan and nano-chitosan showed promising antimicrobial activity against *S.aureus*, *Pseudomonas. aeruginosa*, *S.* Typhimurium, and *E. coli*. It was also seen that nano-chitosan had more activity than chitosan because of their properties (Divya et al., 2017). Biodegradable films loaded with essential oils are novel techniques to improve food safety and extend the shelf life of foods by direct and/or indirect contact (Du et al., 2015).

This study aims to estimate the antibacterial of various natural agents such as essential oils, and their mixtures, chitosan, nano-chitosan, and chitosan film enriched with EOs against multidrug-resistant pathogenic bacteria.

Materials and methods

Pathogenic bacterial strains

Five pathogenic bacterial strains, including *Salmonella* Typhimurium ATCC 25566 and *Staphylococcus aureus* ATCC 6538 were purchased from the Microbiological Resource Center (MERCIN) at Faculty of Agriculture, Ain Shams University, Cairo, Egypt while *S.* Typhimurium ATCC14028, *Staph. aureus* ATCC 6538P, and *Staph. aureus* ATCC 20231 were purchased from the Microbiological Laboratory of Animal Health Institute, Cairo, Egypt. The test bacteria were grown on Mueller Hinton agar MHA **(Jabbari et al., 2010)** then cultured in tryptone soy broth TSB **(Roberts and Greenwood, 2003)** at 37ºC for 24 h and kept at 4ºC for further experiments.

Antibiotics

Twenty common antibiotics used in medical practice belonging to different groups were purchased from Oxoid, UK., and are shown in **Table** (1).

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*).

Chitosan and nano-chitosan characterization

Chitosan powder (molecular weight: 100-300 KDa; degree of deacetylation: 75) was obtained from ACROS ORGANICS (Belgium). While nano-chitosan (size: 50–100 nm) was purchased from Nano-Fab Technology, New Maadi, Cairo.

Inoculum preparation

A loopful of each tested pathogenic bacteria were 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. The cells were harvested, washed, and then suspended to a final cell density of 12 x 10^6 CFU/ml.

Antibiotic sensitivity by disk diffusion test

One milliliter of each bacterial inoculum (10^6 CFU) was streaked on sterile Petri dishes containing Muller and Hinton Agar (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 (depending on the inhibition zone) as sensitive, intermediate, and resistant according to Clinical Laboratory Standard Institute**(CLSI., 2015)**.

Antibacterial activity of some chemical preservatives

Different concentrations of preservatives were prepared by dissolving them in Mueller Hinton broth (MHB) **(Jabbari et al., 2010)**. Those preservative 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 trisodium phosphate and sodium lactate at the same concentrations were 1%, 2%, and 3%. The multidrug-resistant pathogenic bacteria were inoculated individually in Petri dishes containing tryptic soy agar medium **(Roberts et al, 1995)**. Then, preservative impregnated disks 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)**.

Antibacterial activity of EOs

One milliliter of the most antibiotic-resistant bacterial inoculum was spread onto sterile MHA supplemented with tween 80% (0.01% v/v). 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 by**(López et al., 2005)**. Commercially available gentamicin disk (30µg) was used as a positive control. The inhibition zone was determined in millimeters.

Minimum inhibitory concentration (MIC) for EOs

The most effective EOs 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 MHB **(Jabbari et al., 2010)** and mixed with 50-500 µl/100 ml of each EO or each combinations **(Moreira et al, 2005)** supplemented with tween 80% (0.01% v/v) and then incubated at 37˚C for 24 h. MIC was defined as the concentration that completely inhibited the visible growth of bacteria in broth medium and was confirmed by re-inoculating on MHA **(Berche and Gailard 1996).**

Synergistic effect of EOs combination

The synergistic effect of EO combinations was estimated by determining the fractional inhibition concentration (FIC) index for each combination using the following equations **(Davidson and Parish 1988)**:

$$FIC_1 = \frac{\text{MIC of A/B}}{\text{MIC of a}}$$

$$FIC_2 = \frac{\text{MIC of A/B}}{\text{MIC of b}}$$

$FIC = FIC_1 + FIC_2$, A/B = combination oil, a/b = individual oil

FIC index < 1: synergistic effect, = 1: additive effect, > 1: antagonistic effect

Antibacterial activity of chitosan and nano-chitosan

Briefly, 9-mm wells were punched over the agar plates. Chitosan (2g) and nano-chitosan (2mg) 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/well were placed in the wells. These plates were kept at room temperature for 1h 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).**

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 **(Baron et al., 1994).**

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).**

Preparation of EO-loaded chitosan films

The chitosan films were prepared by dissolving chitosan in an aqueous solution with glacial acetic acid (1% w/v) and then stirring on a magnetic stirrer hot plate at 50˚C. The MICs of effective individual and EOs combinations 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., 2012a).**

Antibacterial effect of EO-loaded chitosan films

Disks (12 mm) of films were cut 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).**

Antioxidant activity of EO-loaded chitosan film

The percentage of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was calculated using the following equation: DPPH scavenging effect (%) = $Abs_{DPPH} - Abs_{Extract}/Abs_{DPPH} \times 100$

Where Abs_{DPPH} is the absorbance value at 517 nm of the methanolic solution of DPPH, and $Abs_{extract}$ is the absorbance value at 517 nm of sample extracts **(Siripatrawana and Harteb, 2010)**.

Total phenols

Total phenols were determined according to the method described previously **(Singleton et al., 1999)**.

Results and discussions

Sensitivity of pathogenic bacterial strains to commercial antibiotics

S. Typhimurium ATCC 25566 was resistant to 80% while *S.* Typhimurium ATCC 14028 was resistant to 65% of the tested antibiotics in **Table** 1. *S. Typhimurium* ATCC 25566 and *S.* Typhimurium ATCC 14028 were sensitive to gentamycin which belongs to the aminoglycoside group. This is due to aminoglycosides being one of the three major classes of antibiotics, are highly bactericidal, and are commonly used to treat serious Gram-negative bacterial infections **(Fair and Tor, 2014).**

Despite Penicillin, Ampicillin, Vancomycin, Ciprofloxacin, and colistin, has antibacterial activity against Gram-negative bacteria, the two bacterial strains showed resistance to those antibiotics. This can be explained by different mechanisms of antimicrobial resistance in Gram-negative bacteria including target modification, efflux pumps, hydrolyzing enzymes such as β-lactamases which hydrolyze β-lactam ring present in penicillins, β-lactam and β-lactamase inhibitor combinations, cephalosporins, monobactam, and carbapenems **(Parajuli et al., 2017).**

Staph. aureus ATCC 6538P , *Staph. aureus* ATCC 6538and *Staph. aureus* ATTC2023 were resistant to (70% ,80%and 35%, respectively) **Table** 2.

Although amikacin and gentamicin were belonging to the aminoglycoside group, *Staph. aureus* ATCC 6538P and *Staph. aureus* ATCC 6538 were sensitive to the first one and show intermediate sensitivity to the second one. Whereas *Staph. aureus* ATCC 20231was sensitive to both antibiotics belonging to aminoglycoside.

Penicillin, ampicillin, rifampicin, and nitrofurantoin were not effective against most Gram-positive and Gram-negative bacteria. This may be due to excessive use of antibiotics has accelerated the development of methicillin resistance, and resistance in *Staph. aureus* can be explained by mutation or modification of antibiotic targets, inactivation of β-lactam antibiotics by β-lactamase, a reduction in membrane permeability, or increased activity of efflux pumps **(Lade & Kim, 2021).**

Table 1. Sensitivity of *Salmonella* Typhimurium to commercial antibiotics.

Antibiotics	Disk content µg/mL	*Salmonella* Typhimurium (ATCC 14028)		*Salmonella* Typhimurium (ATCC 25566)	
		I.Z (mm)	I.S	I.Z (mm)	I.S
Penicillin	10	11.5± 0.5	R	10.0± 0.3	R
Ampicillin	10	11.0± 0.1	R	11.6± 0.5	R
Amoxicillin+ Clavulanic acid	30	11.7± 0.6	R	10.5± 0.7	R
Cephalexin g1	30	15.0± 0.5	R	16.5± 0.4	R
Ceftriaxone g3	30	11.7± 0.7	R	12.4± 0.3	R
Cefaclor g2	30	10.5± 0.3	R	N.I	R
Ceftazidime g3	30	10.5± 0.4	R	N.I	R
Rifampicine	5	18.5± 0.5	I	10.8± 0.7	R
Vancomycin	30	7.5± 0.2	R	7.0± 0.3	R
Azithromycin	15	13.0± 0.3	S	10.5± 0.5	R
Amikacin	10	16.0± 0.5	I	13.8± 0.3	R
Gentamicin	10	17.0± 0.2	S	15.0± 0.4	S
Oxytetra acid	10	15.0± 0.4	R	13.5± 0.5	R
Doxycycline	30	11.8± 0.3	I	11.0± 0.6	I
Colistin	10	1.5± 0.2	R	3.0± 0.4	R
Sulfamethoxazole	30	14.0± 0.5	I	16.0± 0.3	S
Cidocetine	30	13.0± 0.6	I	12.5± 0.2	I
Ciprofloxacin	5	15.0± 0.5	R	15.0± 0.1	R
Levofloxacin	5	9.5± 0.6	R	N. I	R
Nitrofurantoin	30	N.I	R	N.I	R

R, Resistant; I, Intermediate; S, Sensitive; CLSI, Clinical Laboratory Standards Institute; N.I, No Inhibition; I.Z, Inhibition zone; I.S, Interpretive standard.

Table 2. Sensitivity of *Staphylococcus aureus* to commercial antibiotics.

Antibiotics	Disk content µg/mL	*Staphylococcus aureus* (ATCC 6538P)		*Staphylococcus aureus* (ATCC 6538)		*Staphylococcus aureus* (ATCC 20231)	
		I.Z (mm)	I.S	I.Z (mm)	I.S	I.Z (mm)	I.S
Penicillin	10	N.I	R	6.0 ±0.3	R	N.I	R
Ampicillin	10	7.5 ± 0.3	R	9.0 ± 0.4	R	10.0 ± 0.4	R
Amoxicillin+ Clavulanic acid	30	10. 5 ± 0.4	R	13.0 ± 0.1	R	25.0 ± 0.6	S
Cephalexin g1	30	13.0 ± 0.7	I	13.0 ± 0.4	R	20.5 ± 0.5	S
Ceftriaxone g3	30	12.0 ± 0.4	R	11.3 ± 0.3	R	18.5 ± 0.5	I
Cefaclor g2	30	N.I	R	N.I	R	N.I	R
Ceftazidime g3	30	10.0 ± 0.6	R	N.I	R	12.0 ± 0.2	R
Rifampicine	5	13.0 ± 0.7	R	12.8 ± 0.6	R	15.5 ± 0.4	R
Vancomycin	30	11.5 ± 0.2	I	10.8 ± 0.5	I	17.0 ± 0.1	S
Azithromycin	15	10.5 ± 0.5	R	11.5 ± 0.2	R	19.0 ± 0.6	S
Amikacin	10	18.0 ± 0.7	S	16.5 ± 0.3	S	21.0± 0.7	S
Gentamicin	10	14.0 ± 0.1	I	13 ± 0.4	I	16.5 ± 0.5	S
Doxycycline	30	12.8 ± 0.5	I	12.0 ± 0.4	R	14.0± 0.4	I
Oxytetra acid	10	13.0 ± 0.6	R	12.5 ± 0.8	R	19.5 ± 0.5	S
Colistin	10	N.I	R	N.I	R	11.5 ± 0.2	R
Sulfamethoxazole	30	15.0 ± 0.4	I	14.0 ± 0.5	I	19.5 ± 0.5	S
Ciprofloxacin	5	16.0 ± 0.3	I	15.0 ± 0.6	R	20.5 ± 0.1	S
Levofloxacin	5	11.0 ± 0.5	R	N.I	R	20.0 ± 0.2	S
Cidocetine	30	11.0 ± 0.6	R	11.4 ± 0.8	R	18.5 ± 0.2	S
Nitrofurantoin	30	10.0 ± 0.5	R	N.I	R	11.0 ± 0.4	R

R, Resistant; I, Intermediate; S, Sensitive; CLSI, Clinical Laboratory Standards Institute; N.I, No Inhibition; I.Z, Inhibition zone; ± , Standard Deviation; I.S, Interpretive standard.

Table 3. Inhibition zone of concentration sodium benzoate, sodium nitrite, sodium tripolyphosphate, and sodium lactate for pathogenic bacteria.

Preservatives	*Salmonella* Typhimurium (ATCC 25566)	*Staphylococcus aureus* (ATCC 6538)
	Inhibition zone (mm)	
Disc saturated sterile water (control)	N.I	N.I
Sodium benzoate (mg/ml)		
1.00	5.3 ±0.5	12.0± 0.4
1.25	6.8 ±0.3	14.3± 0.5
1.50	9.3 ±0.1	13.0± 0.3
Sodium nitrite (mg/ml)		
1.0	11.0 ±0.2	12.0± 0.4
1.5	12.8 ±0.1	13.2± 0.5
2.0	14.6 ±0.2	16.0± 0.2
Sodium tripolyphosphate (%)		
1.0	8.0 ±0.1	10.3± 0.7
2.0	9.3 ±0.1	11.7± 0.2
3.0	10.5 ±0.4	13.4± 0.5
Sodium lactate (%)		
1.0	10.0 ±0.2	12.0± 0.4
2.0	11.6 ±0.2	13.4± 0.3
3.0	11.3 ±0.4	15.4± 0.2

Antibacterial activity of preservatives

Chemical preservatives showed varying inhibition zones depending on the concentration of the preservative employed and the type of tested bacterial strains as shown in **Table** 3 according to **(Saranraj et al., 2012).**

Sodium nitrite at 2.0 mg/ml gave the highest inhibition zone against *Staph. aureus* followed by *S.* Typhimurium with inhibition zone 16.0 and 14.6 mm, respectively. This is an indication that two tested bacterial strains were intermediate to the chemical preservatives as reported by **(Selim et al., 2012)** who found that the zone of inhibition lesser than (≤ 14mm) is resistant, (15-19mm) are intermediate while the zone of inhibition more than (≥20mm) is sensitive to chemical food preservatives.

Sodium lactate and sodium tripolyphosphate showed a higher inhibition zone for *Staph. aureus* than *S.* Typhimurium. These results are in harmony with **(Moon et al., 2011)**. The effect of the sodium

tripolyphosphate for tested pathogenic bacterial may be attributed due to the sequestration of metal ions in the cell wall which leads to loss of cell wall integrity, thus inhibiting the growth of microorganisms **(Kataria et al., 2020).**

Sodium benzoate at 1.5 mg/ml gave the lowest inhibition zone against *S.* Typhimurium *Staph. aureus* (9.3, and 13.0 mm, respectively) compared to other chemical preservatives On the other hand **(Gyawali et al., 2015)** revealed that the most effective chemical preservative was sodium benzoate (53.3%).

Antibacterial activity of tested essential oils

Essential oils of turmeric, marjoram, cumin, and black pepper did not form any inhibition zone against *S.*Typhimurium while against *Staph. aureus* gave inhibition zone11.5,10.0 and 10.5 mm, respectively. These results are in agreement with those obtained by **(Tariq et al., 2019)** who confirmed that Gram-positive and Gram-negative bacteria diff er in their sensitivity to EOs.

Cinnamon, clove, and thyme oils gave the maximum values of inhibition zones against the two tested pathogenic bacterial strains compared to other oils. This is in the line with **(Das et al., 2012).** The antibacterial activity of EOs occurs by easily disrupt the cell membrane and make it more permeable **(Khorshidian et al., 2018)** moreover, they interruption transport processes and interact with trans membrane proteins and other compounds within the cell. EOs also have adverse eff ects on enzymes **(Hu et al., 2017).**

MIC of essential oils against pathogenic bacteria

Cinnamon and clove oils possessed an important antibacterial effect against both *S.* Typhimurium and *Staph. aureus* at MIC (350 and 250µl/100ml, respectively) as shown in **Table** 5. This may be due to the ability of these essential oils to penetrate the membranes of bacteria, leading to their lysis **(Vani and Lakshmi, 2014)**. MIC of garlic oil against *S.* Typhimurium and *Staph. aureus* at 500 and 400 µl/100ml, respectively whilst the inhibition of *S.* Typhimurium and *Staph. aureus* was observed at 350-400 µl/100ml, respectively when used thyme oil. The antibacterial activity of thyme oil has been largely attributed to antimicrobial effects in addition to antioxidant features **(Nikolić et al., 2014).**

Table 4. Antibacterial activity of essential oils against pathogenic bacteria.

Essential oils	*Salmonella Typhimurium* (ATCC 25566)	*Staphylococcus aureus* (ATCC 6538)
	Inhibition zone (mm)	
Thyme	18.0 ± 0.2	23.0 ± 0.5
Turmeric	N.I	11.5 ± 0.4
Parsley	12.0 ± 0.3	15.5 ± 0.2
Garlic	17.5 ± 0.5	20.3 ± 0.7
Cumin	N.I	10.0 ± 0.4
Clove	24.6 ± 0.1	26.0 ± 0.2
Pepper black	N.I	10.5 ± 0.4
Ginger	11.8 ± 0.2	12.0 ± 0.4
Cinnamon	23.5 ± 0.2	26.5 ± 0.2
Marjoram	N.I	N.I
Gentamycin (30µg/mL)	16.0 ± 0.5	15.0 ± 0.4

N.I (NO Inhibition) <9 mm diameter.

MIC of the four EOs on *Staph. aureus* less than *S.* Typhimurium, this was probably due to the protective effect of the outer membrane of Gram-negative bacteria to the hydrophobic antimicrobial compound **(Thongson et al., 2005)**.

Effect of combinations essential oils

The mixture of cinnamon and clove oils showed the highest inhibition zone against *Staph. aureus* and *S.* Typhimurium compared to other mixtures **Table** 6. The interpretation synergistic antimicrobial cinnamon/clove oil combination might be due to the interactions between the main constituents of the oils **(Purkait et al., 2020).** The combination of cinnamon and thyme oils showed a higher inhibition zone for *Staph. aureus* than *S.* Typhimurium. These results are in agreement with those obtained by **(El Atki et al., 2020)** who found that a combination of cinnamon and thyme oils showed synergistic activity against *Staph. aureus*.

Based on the FIC index in **Table** (6) all combinations showed a synergistic effect except thyme and garlic oils combination which exhibited an additive effect against both selected bacterial strains. Regarding MIC, a mixture of cinnamon and clove EOs (1:1, v/v) exhibited a clear synergistic effect against *Staph. aureus* and *S.* Tphymurium since the lowest values of MIC 150 µl/100ml. Whereas the mixture of (thyme + garlic) EOs revealed an additive effect against *S.* Typhimurium and *S. aureus* since the highest values of MIC 250 µl/100ml.

The higher efficacy of oil combinations compared with individual oils might be attributed to each essential oil has its unique chemical components which may possess varying modes of action. This

enhances the likelihood of reducing a microbe's potential resistance according to the previous study **(El Atki et al., 2020).**

Antibacterial activity of chitosan and nano-chitosan

Chitosan at 100 µL/well showed strong antibacterial activity against both *Staph*. *aureus* and *S.* Typhimurium in **Table** 7. These results are in agreement with **(Jolly & Menon 2015).** On the other hand, MIC of chitosan showed the same concentration for both bacteria (128 µg/ml).

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

Bacterial strains	Values of MIC for essential oils (µl/100ml)			
	Clove	Thyme	Cinnamon	Garlic
Salmonella Typhimurium (ATCC 25566)	350 ± 3.0	400 ± 5.0	350 ± 5.0	500 ±7.0
Staphylococcus aureus (ATCC 6538)	250 ± 5.0	350 ± 2.0	250 ± 5.0	400 ± 4.0

Table 6. Effect of essential oils combination against pathogenic bacteria.

Bacterial strains	Essential oils mixture	Inhibition zone of mixture oils (mm)	MIC of mixture oils (µl/100ml)	FIC (index)	Effect of combination
Salmonella Tphymurium (ATCC 25566)	Cinnamon + Clove	30.5 ± 0.2	150 ± 2.0	0.87	synergistic
	Cinnamon + Garlic	28.6 ± 0.5	170 ± 4.0	0.90	synergistic
	Cinnamon + Thyme	28.8 ± 0.3	190 ± 9.0	0.96	synergistic
	Clove + Thyme	30.0 ± 0.5	200 ± 1.0	0.94	synergistic
	Clove + Garlic	25.0 ± 0.4	200 ± 5.0	0.90	synergistic
	Thyme + Garlic	21.0 ± 0.3	250 ± 4.0	1.0	additive
Staphylococcus aureus (ATCC 6538)	Cinnamon + Clove	33.5 ± 0.1	150 ± 5.0	0.87	synergistic
	Cinnamon + Garlic	30.6 ± 0.4	170 ± 8.0	0.90	synergistic
	Cinnamon + Thyme	31.8 ± 0.5	190 ± 6.0	0.96	synergistic
	Clove + Thyme	31.6 ± 0.4	200 ± 5.0	0.94	synergistic
	Clove + Garlic	25.0 ± 0.3	200 ± 7.0	0.90	synergistic
	Thyme + Garlic	21.0 ± 0.4	250 ± 4.0	1.0	additive

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

Antibacterial agents	Bacterial strains	
	Salmonella Typhimurium (ATCC 25566)	*Staphylococcus aureus* (ATCC 6538)
Chitosan/plate (µl/ml)	Inhibition zone (mm)	
25	18.0 ± 0.4	20.5 ± 0.4
50	19.0 ± 0.3	22.0 ± 0.5
75	25.0 ± 0.5	25.0 ± 0.3
100	26.8± 0.2	28.0 ± 0.2
MIC (µg/ml)	128 ± 3.0	128 ± 0.7
Nano-chitosan/plate (µl/ml)	Inhibition zone (mm)	
25	18.5 ± 0.2	25.3 ± 0.5
50	22.0 ± 0.3	27.0 ± 0.1
75	25.0 ± 0.4	27.0 ± 0.3
100	29.3 ± 0.5	30.0 ± 0.2
MIC (µg/ml)	51.2 ± 5.0	25.6 ± 0.6

Nano-chitosan at 100µL/well showed a maximum inhibition zone (30.0 mm) against *S*. *aureus* followed by *S*. Typhimurium 29.3 mm. Additionally, nano-chitosan showed higher antimicrobial activity than chitosan against the tested pathogenic bacteria strains. This may be due to the features of nano-chitosan such as small size and increased surface area **(Rozman, et al., 2019).** Regarding the MIC of nano-chitosan, *Staph. aureus* was more sensitive to nano-chitosan with a lower MIC value than *S*. Typhimurium at (25.6 and 51.2 µg/ml, respectively). This may be due to the outer membrane consisting of lipopolysaccharides, lipoproteins, and phospholipids which act as a potential barrier against the entry of foreign molecules into the cell wall **(Abou-Zeid et al., 2010).**

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

Antibacterial agents (µl/ml)	Bacterial strains	
	Salmonella Typhimurium (ATCC 25566)	*Staphylococcus aureus* (ATCC 6538)
	Inhibition zone (mm)	Inhibition zone (mm)
Chitosan + garlic	28.3 ± 0.5	30.0 ± 0.2
Chitosan + thyme	32.0 ± 0.4	37.2 ± 0.4
Chitosan + cinnamon	28.6 ± 0.5	32.0 ± 0.2
Chitosan + clove	29.0 ± 0.3	34.5 ± 0.5
Chitosan + (cinnamon +clove)	34.0 ± 0.2	38.5 ± 0.3
Chitosan + (clove+thyme)	37.0 ± 0.3	42.5 ± 0.5
Nano-chitosan + garlic	15.0 ± 0.1	19.8 ± 0.2
Nano-chitosan + thyme	20.0 ± 0.2	24.0 ± 0.4
Nano-chitosan + cinnamon	15.0 ± 0.4	21.0 ± 0.5
Nano-chitosan + clove	21.0 ± 0.1	25.8 ± 0.3
Nano-chitosan + (cinnamon + clove)	22.0 ± 0.3	25.0 ± 0.2
Nano-chitosan + (clove + thyme)	20.0 ± 0.2	32.5 ± 0.2

Antibacterial activity of chitosan and nano-chitosan combined with essential oils

Chitosan or nano-chitosan incorporated with EOs exhibited potent antibacterial e-ects against the two tested bacteria with a variable degree as shown in **Table** 8. Chitosan combined with cinnamon and clove oils had a synergistic antibacterial effect against *Staph .aureus* and S. Typhimurium with inhibition zone (38.5 and 34.0 mm, respectively). This result is in line with that reported previously by **Purkait et al., 2020.**

Chitosan showed the strongest activity and the growth inhibition of both *S.aureus* and *S.* Typhimurium when enriched with thyme and clove EO together according to **(Batiha et al., 2020)** who found that clove oil has both acetate, eugenol, and β-caryophyllene, which are all considered significant phytochemicals and antioxidant features. In addition to the mode of action chitosan by binding to the negatively charged bacterial cell wall to disrupt by altering cell membrane permeability, and later by attaching to the DNA to inhibit its replication, leading to cell death.

The lowest inhibition zones were observed for chitosan enriched with garlic oil against *S.* Typhimuṛium and *S. aureus* compared to chitosan combined with other oils. On the opposite **Jolly & Menon 2015,** proved that garlic oil has an antibacterial effect, as this oil is rich in organ sulfur compounds which inhibit the growth of several bacteria such as *E. coli* and *S. aureus*. Chitosan enriched with garlic, thyme, cinnamon, and clove oils revealed a higher inhibition zone against *S.* Typhimuṛium and *Staph. aureus* than nano-chitosan enriched with the same oils. These results may be attributed to when nano-chitosan was mixed with oils, it turned into a particle. Our results are supported by **Hosseini et al., 2013** who found that chitosan can bind and improve the bioactive components and bactericidal activities of EOs.

Total phenolic content (TPC) and antioxidant activity DPPH in biodegradable chitosan film

Total phenolic content increase by using any oil with chitosan film. Maximum phenolic content values were observed in chitosan film enriched with clove and thyme oils combination followed by the combination of cinnamon and clove oils in **Table** 9. These results are in harmony with (**Shaaban and Khaled, 2014**) who found that the total phenolic content in the chitosan film increased with essential oils.

As regards the antioxidant activity, the lowest value was observed in chitosan film enriched with cinnamon oil whereas chitosan film enriched with clove and thyme oils gave the highest one 93%. Our results were supported by **(Ballester-Costa et al., 2016)** who found the chitosan films without EO showed a slight scavenging activity on DPPH while chitosan film added with thyme essential oils had the highest antioxidant activity.

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

Antibacterial agents	Total phenol content (mg/m)	Antioxidant activity DPPH (%)
Chitosan film (control)	0.00	42.3 ± 0.5
Chitosan film + thyme	6.52 ± 0.3	74.0 ± 0.4
Chitosan film + cinnamon	5.43 ± 0.2	71.7 ± 0.2
Chitosan film + clove	5. 50 ± 0.2	79.6 ± 0.1
Chitosan film+ (cinnamon+ clove)	7. 34 ± 0.1	89.8 ± 0.3
Chitosan film + (clove + thyme)	8.01 ± 0.4	93.0 ± 0.5

Compounds in EOs have shown that the antioxidant activities may be due to the redox properties exerted by various possible mechanisms: free radical scavenging activity, hydrogen donors, and transition metal chelating activity **(Liyana-Pathirana and Shahidi, 2006**)

Antibacterial activity of biodegradable chitosan–film loaded with EOs

Chitosan film incorporated with thyme oil showed a high inhibition zone (35.7 and 30.0mm) against *Staph. aureus* and *S.* Typhimurium, respectively as shown in **Table** 10. The antibacterial activity of chitosan films loaded with thyme EO could be attributed to action thymol and these compounds exhibit antimicrobial activity against a broad spectrum of both Gram-negative and Gram-positive bacteria **(Venkatachalam and Lekjing, 2020).** Chitosan films loaded separately with clove oil and cinnamon oil showed the lowest inhibition zone against *S.* Typhimurium and *Staph. aureus* compared to other chitosan films loaded with oils.

Chitosan film enriched with a mixture of (clove + thyme) showed strong antibacterial activity than chitosan incorporated with a mixture of (cinnamon+clove) against *Staph. aureus* and *S.*Typhimurium. This result is in agreement with that reported previously by **(Mehdizadeh et al., 2012)** who proved that thyme EO incorporated with chitosan films exhibited a synergistic effect with high inhibition levels against *Staph. aureus* and *S.* Typhimurium. Chitosan films enriched with essential oils showed effective antibacterial activity against tested pathogenic bacterial strains whereas chitosan films oil-free did not demonstrate antibacterial activity. This may be explained by the fact that the chitosan molecules were fixed inside the film and could not diffuse into the surrounding agar medium **(Wang et al., 2011).**

Conclusion and recommendation

According to the results obtained, chitosan solution and biodegradable film loaded with EOs are more effective than utilizing oils, chitosan, or nano-chitosan separately as antibacterial activity against pathogenic bacteria. Using chitosan loaded with EOs for food preservation might thus be recommended.

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)					
Salmonella Typhimurium (ATCC 25566)	N.I	30.0 ± 0.3	26.0 ± 0.2	26.6 ± 0.4	34.0 ± 0.5	36.0 ± 0.2
Staphylococcus aureus (ATCC 6538)	N.I	35.7± 0.5	32.0 ± 0.3	32.0 ± 0.2	37.8 ± 0.3	39.5 ± 0.4

N.I (NO Inhibition around dick

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

All authors declare their consent for publication.

Contribution of authors

This study was designed and implemented by all the authors, where all contributed to writing the manuscript, interpreting information presented, and have read and agreed to the final version of the manuscript.

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References

Abou-Zeid, N.Y., Waly, A.I., Kandile, N., Rushdy, A., El-Sheikh, M., Ibrahim, H., 2010. Preparation, characterization and antibacterial properties of cyanoethylchitosan/cellulose acetate polymer blended films. Carbohydr. Polym. 84, 223. https://doi.org/10.1016/j.carbpol.2010.11.026.

Aliasghari, A., Khorasgani, M.R., Vaezifar, S., Rahimi, F., Younesi, H., Khoroushi, M., 2016. Evaluation of antibacterial efficiency of chitosan and chitosan nanoparticles on cariogenic streptococci: An in vitro study. Iran. J. Microbiol. 8, 93–100. https://doi.org/10.5281/zenodo.3342597.

Aween, M.M., Hassan, Z., Huda-Faujani, N., Emdakim, M.M., Muhialdin, B.J., 2014. Potency of honey as antibacterial agent against multiple antibiotic resistant pathogens evaluated by different methods. Am. J. Appl. Sci.11(10) 1773-1783 https://doi.org/10.3844.

Awuchi, C.G., Twinomuhwezi, H., Igwe, V.S., 2020. Food Additives and Food Preservatives for Domestic and

Industrial Food Applications. J. Anim. 2(1), 1–16.

Ballester-Costa, C., Sendra, E., Fernández-López, J., Viuda-Martos, M., 2016. Evaluation of the antibacterial and antioxidant activities of chitosan edible films incorporated with organic essential oils obtained from four *Thymus* sp. J. Food Sci. Technol. 53, 3374–3379. https://doi.org/10.1007/s13197-016-2312-y.

Baron, E.J., Peterson, L.R. & Finegold, S.M., 1994. Bailey & Scott's Diagnostic Microbiology. 9th Edition. St. Louis: Mosby.

Batiha, G.E.-S., Alkazmi, L.M., Wasef, L.G., Beshbishy, A.M., Nadwa, E.H., Rashwan, E.K., 2020. *Syzygium aromaticum* L. (Myrtaceae): Traditional uses, bioactive chemical constituents, pharmacological and toxicological activities. Biomolecules 10 (2), 202. https://doi.org/10.3390/biom10020202.

Bauer, A.W., Kirby, W.M., Sherris, J.C., Turck, M., 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45, 493–496.

Berche P, Gaillard, J., M, S., (1996) Bactériologie : bactéries des infections humaines - Ghent University Library. 64-71.

Bintsis, T. (2017) Foodborne pathogens. In *AIMS Microbiology* 3 (3), 529-563. https://doi.org/10.3934/microbiol.2017.3.529.

Bintsis, T., 2017. Foodborne pathogens. AIMS Microbiol. 3 (3), 529-563 https://doi.org/10.3934/microbiol.2017.3.529.

Bondi, M., Lauková, A., De Niederhausern, S., Messi, P., Papadopoulou, C., 2017. Natural preservatives to improve food quality and safety. J. Food Qual.2017, 1-3 https://doi.org/10.1155/2017/1090932.

Chi, S., Zivanovic, S., Penfield, M.P., 2006. Application of chitosan films enriched with oregano essential oil on bologna - Active compounds and sensory attributes. Food Sci. Technol. Int. 12, 111–117. https://doi.org/10.1177/1082013206063845.

Clinical and Laboratory Standards Institute (CLSI), 2015. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement Clinical and Laboratory Standards Institute. CLSI Doc. M100-S16CLSI, Wayne, PA.

CLSI., 2017. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Seventh Informational Supplement. CLSI Document M100-S27. CLSI. Perform. Stand. Antimicrob. Susceptibility Testing. 27th ed. CLSI Suppl. M100. Wayne, PA Clin. Lab. Stand. Institute; 2017.

Das, M., Rath, C.C., Mohapatra, U.B., 2012. Bacteriology of a most popular street food (Panipuri) and inhibitory effect of essential oils on bacterial growth. J. Food Sci. Technol.49, 564-571 https://doi.org/10.1007/s13197-010-0202-2.

Davidson, P.M., Parish, M.E., 1988. Methods for testing the efficacy of food antimicrobials. Food Technol. 43, 148–155.

Delcour, A.H., 2009. Outer membrane permeability and antibiotic resistance. Biochim. Biophys. Acta 1794, 808–816. https://doi.org/10.1016/j.bbapap.2008.11.005.

Divya, K., Vijayan, S., George, T.K., Jisha, M.S., 2017. Antimicrobial properties of chitosan nanoparticles: Mode of action and factors affecting activity. Fibers Polym. 18, 221–230. https://doi.org/10.1007/s12221-017-6690-1.

Du, W.X., Avena-Bustillos, R.J., Sheng, S., Hua, T., McHugh, T.H., 2015. Antimicrobial volatile essential oils in edible films for food safety. Sci. against Microb. Pathog. Commun. Curr. Res. Technol. Adv. 2(1), 1124–1134.

El Atki, Y., Aouam, I., El Kamari, F., Taroq, A., Lyoussi, B., Oumokhtar, B., Abdellaoui, A., 2020. Phytochemistry, antioxidant and antibacterial activities of two *Moroccan Teucrium polium* L. subspecies: Preventive approach against nosocomial infections. Arab. J. Chem. 13, 3866-3874. https://doi.org/10.1016/j.arabjc.2019.04.001.

Fair, R.J., Tor, Y., 2014. Antibiotics and bacterial resistance in the 21st century. Perspect. Medicin. Chem. 6, 25–64. https://doi.org/10.4137/PMC.S14459.

Giacometti, F., Shirzad-Aski, H., Ferreira, S., 2021. Antimicrobials and food-related stresses as selective factors for antibiotic resistance along the farm to fork continuum. Antibiotics.10(6),2-29. https://doi.org/10.3390/antibiotics10060671.

Gyawali, R., Hayek, S.A., Ibrahim, S.A., 2015. Plant extracts as antimicrobials in food products: Mechanisms of action, extraction methods, and applications. In: Handbook of Natural Antimicrobials for Food Safety and Quality. 49–68. https://doi.org/10.1016/B978-1-78242-034-7.00003-7.

Hosseini, S.F., Zandi, M., Rezaei, M., Farahmandghavi, F., 2013. Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: Preparation, characterization and in vitro release study. Carbohydr. Polym. 95, 50–56. https://doi.org/10.1016/j.carbpol.2013.02.031.

Hu, Y., Zhang, J., Kong, W., Zhao, G., Yang, M., 2017. Mechanisms of antifungal and anti-aflatoxigenic properties of essential oil derived from turmeric (*Curcuma longa L.*) on *Aspergillus flavus*. Food Chem. 220, 1–8. https://doi.org/10.1016/j.foodchem.2016.09.179.

Jabbari Nooghabi, M., Jabbari Nooghabi, H., and Nasiri, P.

(2010). "Detecting outliers in gamma distribution." Communications in Statistics-Theory and Methods, 39: 698–706.

Jolly, D., K, V.M., 2015. Antibacterial Effect of Garlic and Ginger Extracts on *Escherichia coli* and *Listeria monocytogenes*. Int. J. Appl. Pure Sci. Agric. 1(2), 111–118.

Khorshidian, N., Yousefi, M., Khanniri, E., Mortazavian, A.M.,N.D., 2018 Potential application of essential oils as antimicrobial preservatives in cheese. Innov. Food Sci. Emerg. Technol.45, 62-72 https://doi.org/10.1016/j.ifset.2017.09.020.

Lade, H. and Kim, J. S. (2021) Bacterial targets of antibiotics in methicillin-resistant *Staphylococcus aureus*. Antibiotics, 10(4), 398. https://doi.org/10.3390/ antibiotics10040398.

Liyana-Pathirana, C.M., Shahidi, F., 2006. Antioxidant properties of commercial soft and hard winter wheats (*Triticum aestivum* L.) and their milling fractions. J. Sci. Food Agric.86(3), 477-485. https://doi.org/10.1002/jsfa.2374.

López, P., Sánchez, C., Batlle, R., Nerín, C., 2005. Solid- and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. J. Agric. Food Chem. 53, 6939–6946. https://doi.org/10.1021/jf050709v.

Mehdizadeh, T., Tajik, H., Razavi Rohani, S.M., Oromiehie, A.R., 2012a. Antibacterial, antioxidant and optical properties of edible starch-chitosan composite film containing T*hymus kotschyanus* essential oil. Vet. Res. forum an Int. Q. J. 3(3), 167–173.

Moon, J.-H., Park, J.-H., Lee, J.-Y., 2011. Antibacterial action of polyphosphate on *Porphyromonas gingivalis*. Antimicrob. Agents Chemother. 55, 806-812. https://doi.org/10.1128/AAC.01014-10.

Moreira, M., Ponce, A.G., Valle, C.E., Roura, S.I., 2005. Inhibitory parameters of essential oils to reduce a foodborne pathogen. LWT - Food Sci. Technol. 38, 565–570. https://doi.org/10.1016/j.lwt.2004.07.012.

Nikolić, M., Glamočlija, J., Ferreira, I.C.F.R., Calhelha, R.C., Fernandes, Â., Marković, T., Marković, D., Giweli, A., Soković, M., 2014. Chemical composition, antimicrobial, antioxidant and antitumor activity of *Thymus serpyllum* L., *Thymus algeriensis* Boiss. and Reut and *Thymus vulgaris* L. essential oils. Ind. Crops Prod. 52, 183–190. https://doi.org/10.1016/j.indcrop.2013.10.006.

Parajuli, N.P., Acharya, S.P., Mishra, S.K., Parajuli, K., Rijal, B.P., Pokhrel, B.M., 2017. High burden of antimicrobial resistance among gram negative bacteria causing healthcare associated infections in a critical care unit of Nepal. Antimicrob. Resist. Infect. Control 6, 2–9. https://doi.org/10.1186/s13756-017-0222-z.

Purkait, S, Bhattacharya, A., Bag, A., Chattopadhyay, R.R., 2020. Synergistic antibacterial, antifungal and antioxidant efficacy of cinnamon and clove essential oils in combination. Arch. Microbiol. 202, 1439–1448. https://doi.org/10.1007/s00203-020-01858-3.

Roberts, D., and Greenwood, M., 2003. Practical Food Microbiology, Practical Food Microbiology. 2th Edition. London: Public Health Laboratory Service, 2: 55–68. https://doi.org/10.1002/9780470757512.

Rozman, N.A.S., Yenn, T.W., Ring, L.C., Nee, T.W., Hasanolbasori, M.A. and Abdullah, S.Z., (2019) Potential antimicrobial applications of chitosan nanoparticles (ChNP). J. Microbiol. Biotechnol. 29(7), 1009-1013 https://doi.org/10.4014/jmb.1904.04065.

Saranraj, P., (2012) Inhibitory effect of chemical preservatives & organic acids on the growth of bacterial pathogens in poultry chicken. Asian J. Biochem. Pharm. Res. 1(1), 1–13.

Selim, S.A., El Alfy, S.M., Abdel Aziz, M.H., Mashait, M.S., Warrad, M.F., 2012. Evolution of bactericidal activity of selected food additives against food borne microbial pathogens. Biosci. Biotechnol. Res. Asia 9, 7–17. https://doi.org/10.13005/bbra/961.

Seydim, A.C., Sarikus, G., 2007. Antimicrobial activity of whey protein based edible films incorporated with oregano, rosemary and garlic essential oils. Food Res. Int. 39, 639–644. https://doi.org/10.1016/j.foodres.2006.01.013.

Shaaban, H.A., Khaled, F.M., 2014. In-vitro antibacterial and antioxidant properties of starch/ chitosan edible composite film incorporated with thyme essential oil. J. Arab Soc. Med. Res. 9, 54–61.

Singleton, V.L., Orthofer, R., Lamuela-Raventós, R.M., 1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol. 299, 152–178. https://doi.org/10.1016/S0076-6879(99)99017-1.

Siripatrawana, A. and R.Harteb, B., 2010. Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. Food Res. Int. 24, 770–775. https://doi.org/10.1016/j.foodres.2007.04.002.

Stanojević, D., Čomić, L., Stefanović, O., Solujić-Sukdolak, S., 2010. In vitro synergistic antibacterial activity of *Salvia officinalis* L. and some preservatives. Arch. Biol. Sci. 62, 167–174. https://doi.org/10.2298/ABS1001167S.

Tariq, S., Wani, S., Rasool, W., Shafi, K., Bhat, M.A., Prabhakar, A., Shalla, A.H., Rather, M.A., 2019. A comprehensive review of the antibacterial, antifungal and antiviral potential of essential oils and their chemical constituents against drug-resistant microbial pathogens. Microb. Pathog. 134, 103580. https://doi.org/10.1016/j.micpath.2019.103580.

Thongson, C., Davidson, P.M., Mahakarnchanakul, W.,

Vibulsresth, P., 2005. Antimicrobial effect of Thai spices against Listeria monocytogenes and *Salmonella typhimurium* DT104. J. Food Prot. 68, 2054-2058. https://doi.org/10.4315/0362-028x-68.10.2054.

Tong, S.Y.C., Davis, J.S., Eichenberger, E., Holland, T.L., Fowler, V.G.J., 2015. *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin. Microbiol. Rev. 28, 603–661. https://doi.org/10.1128/CMR.00134-14.

Vani, K., Lakshmi, O., 2014. In-vitro antibacterial efficacy of common spices and their effect on permeability of membrane. Int. J. Pharma Bio Sci. 5, 203–211.

Venkatachalam, K., Lekjing, S., 2020. A chitosan-based edible film with clove essential oil and nisin for improving the quality and shelf life of pork patties in cold storage. RSC Adv. 10, 17777–17786. https://doi.org/10.1039/d0ra02986f.

Wang, L., Liu, F., Jiang, Y., Chai, Z., Li, P., Cheng, Y., Jing, H., Leng, X., 2011. Synergistic antimicrobial activities of natural essential oils with chitosan films. J. Agric. Food Chem. 59, 12411–12419. https://doi.org/10.1021/jf203165k.

Xu, X., Biswas, S., Gu, G., Elbediwi, M., Li, Y., Yue, M., 2020. Characterization of multidrug resistance patterns of emerging *Salmonella enterica* serovar rissen along the food chain in China. Antibiotics 9, 1–16. https://doi.org/10.3390/antibiotics9100660.

Zhang, T., Abel, S., Wiesch, P.A., Sasabe, J., Davis, B.M., Higgins, D.E., Waldor, M.K., 2017. Deciphering the landscape of host barriers to *Listeria monocytogenes* infection. Proc. Natl. Acad. Sci. U. S. A. 114, 6334–6339. https://doi.org/10.1073/pnas.1702077114.