JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2015. Vol. **9**(2), p. 1657-1663

Antimicrobial Effect of Oregano and Thyme Essential Oils Coated Carrageenan Based Edible Film

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(Received: 06 April 2015; accepted: 09 June 2015)

Food borne pathogenic bacteria gradually proliferates in meat products during refrigeration storage and deteriorates the food quality. Biopreservatives such as essential oils have capability to prevent the growth of food borne pathogenic bacteria and protects the food from spoilage. Therefore a research was undertaken to study the antimicrobial effect of essential oil coated edible film against *Escherichia coli, Salmonella pullorum, Staphylococcus aureus* and *Listeria monocytogenes*. The levels of essential oil against above test bacteria were optimized on the basis of minimal inhibitory concentration (MIC). In order to assess this, tube dilution method was followed for each essential oil and their combinations. On the basis of experiments, it was found that combination of oregano and thyme essential oil @ 0.02% and 0.03%, respectively showed antibacterial effect against different test bacteria. The results of disc diffusion test revealed that a combined concentration of 0.10% oregano and 0.15% thyme essential oil coated on carrageenan edible film effectively inhibits the growth *Escherichia coli, Salmonella pullorum, Staphylococcus aureus* and *Listeria monocytogenes*.

Key words: Biopreservatives, essential oils, edible film,minimum inhibitory concentration, Tube dilution method, disc diffusion test.

Microbial contamination of ready-to-eat products such as refrigerated meat products and intermediate moisture foods is a serious concern to human[1, 2]. Microbial contaminations in above type of food products occur primarily at the surface. Several traditional methods have been attempted to control microbial growth on food product surface by spray or dips of natural biopreservatives such as essential oils. However, this has limited success because the antimicrobial substances may interact with food components by evaporating or diffusing into bulk food[3, 4]. One new approach to overcome these limitations could be the use of antimicrobial packaging techniques[5] or the application of antimicrobial edible coatings[6] in which the antimicrobial substances are released on the surface of food products with a slow rate and remain at high concentration for extended period of time[7, 8]. The antimicrobial chemicals incorporated into packaging materials contain organic or inorganic acids, metals, alcohols, ammonium compounds or amines[9]. Now a days producers are highly interested in use of natural biopreservatives such as essential oils in antimicrobial packaging because of health concerns of the consumers. Essential oils are rich in phenolic compounds with wide spectrum of antimicrobial activity and categorised as generally recognized as safe (GRAS)[10]. In order to meet consumer demands for more natural products and for packaging materials with low environmental

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impact, many researchers have focused on the incorporation of plant extracts into films, edible coatings and bio-based packaging materials[11,12]. Salient researches include, antimicrobial effect of ê-carrageenan based edible film containing ovotransferrin[13], whey protein isolate film containing oregano oil and their antimicrobial action against spoilage ûora of fresh beef[14], antimicrobial activity of soy edible film incorporated with thyme and oregano essential oils on fresh ground beef patties[15].

Polysaccahrides derived from cellulose, starch, alginate, and their mixtures have been used for edible films most frequently because of their excellent film-forming properties[16]. Carrageenans are water-soluble, galactose polymers extracted from red seaweed. Carageenans are extensively used in food, dairy, and pharmaceutical industries as gelling, emulsifying, and stabilizing agents[17].

Antimicrobial edible films incorporated with volatile antimicrobial compounds such as essential oils can be considered as controlled release systems, and their effectiveness depends on the diffusion of volatile compounds through the edible biopolymer. Therefore, control of the release rates and migration of antimicrobial compounds from films could be a new technique to develop an antimicrobial edible film. The objectives of this work was to determine the concentrations of essential oils as antimicrobial in edible film and the antimicrobial effect of essential oil coated edible film against *Escherichia coli*, *Salmonella pullorum*, *Staphylococcus aureus* and *Listeria monocytogenes*.

MATERIALS AND METHODS

Essential oils

Food grade essential oils like oregano (*Origanum minutiflorum*) and thyme (*Thymus mastichina*) were procured from Shubh Flavour and Fragrances Pvt. Ltd., New Delhi.

Bacterial Strains

Typical meat product bacterial contaminants used in this study were *Escherichia coli, Salmonella pullorum, Staphylococcus aureus* and *Listeria monocytogenes.* They were obtained from the Food Microbiology Lab, Division of Livestock Products Technology, IVRI. The bacterial culture were grown on nutrient agar slants and kept at 4°C. Sub culturing was carried out every month to maintain bacterial viability.

Tube dilution method

Minimal inhibitory concentrations (MIC) were determined by the conventional tube dilution method using brain heart infusion (BHI) broth as a culture media. Each tube containing 5ml BHI broth was inoculated with a loopful of specific bacterial strain and incubated for 24 h at 37°C to enable enough growth of bacteria. 16.66 µl of cultured broth was transferred into another 5ml BHI broth tubes to make 300 times dilution. Then 50 µl of cultured broth from 300 times dilution was transferred into each 5ml BHI broth and arranged in increasing order of essential oil concentrations. All the tubes were incubated for 24 h at 37°C to check MIC. Positive and negative control tubes of 5ml BHI broth were also maintained for result comparison.

Standardized culture plate of test bacterial strain
↓
Transfer one colony of test bacterial strain into 5 ml BHI broth tubes
↓
Incubate these BHI tubes at 37°C for 24 h
↓
From these tubes transfer 16.66 µl to another 5 ml BHI broth tubes to make 300 times dilution
↓
Transfer 50 µl of 300 times diluted broth culture into each 5 ml BHI broth tubes arranged in increasing order of essential oil concentration
↓
Add essential oil in increasing order (0.01 to 0.1%) in order to check MIC
↓
Incubate all the tubes at 37°C for 24-48 h
↓
Check all the tubes for turbidity
↓
The tubes showing no turbidity was the MIC of essential oil against particular bacteria

Application of essential oils in edible film

Mixture of oregano and thyme essential oil was applied on edible film using sterile cotton swab under sterile condition.

Anti microbial properties of edible film containing essential oils

Antimicrobial properties of edible film

containing essential oil were assessed by disc diffusion[18].

Disc diffusion test (zone of inhibition/agar diffusion assay) is a direct contact method using solid medium to measure antimicrobial activity of essential oil in edible films. For disc diffusion tests, edible films with different concentration of essential oil were aseptically cut into approx 12 mm diameter discs and then deposited over the BHI plate inoculated with test bacteria. After 24-48 h of incubation, the inhibition radius around each film disc (colony-free perimeter) was measured. The inhibition area was then calculated in mm^2. Antibacterial activity of carrageenan edible films swabbed with standardized combination of oregano and thyme essential oil were evaluated against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella pullorum and Listeria monocytogenes* using the disc diffusion test.

RESULTS AND DISCUSSION

Optimization of level of oregano, thyme essential oil and their combinations against different test bacteria on the basis of MIC

Oregano, thyme essential oils and their combinations were used for optimization and their levels in edible film were selected on the basis of Minimal Inhibitory Concentration (MIC). In order to assess this, tube dilution method[19] was followed for each essential oil. The antimicrobial activity of essential oils was assessed against *Escherichia coli*, *Salmonella pullorum*, *Staphylococcus aureus* and *Listeria monocytogenes* organisms.

Table 1. Different concentration of oregano essential oil against test bacteria for calculation of minimal inhibitory concentration (MIC)

Essential Oil concentration (%)	*Escherichia coli*	*Salmonella pullorum*	*Staphylococcus aureus*	*Listeria monocytogenes*
0.01	Turbidity	Turbidity	Turbidity	Turbidity
0.02	No Turbidity	Turbidity	No Turbidity	Turbidity
0.03	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.04	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.05	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.06	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.07	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.08	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.09	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.10	No Turbidity	No Turbidity	No Turbidity	No Turbidity

n=6

Table 2. Different concentration of thyme essential oil against test bacteria for calculation of minimal inhibitory concentration (MIC)

Essential Oil concentration (%)	*Escherichia coli*	*Salmonella pullorum*	*Staphylococcus aureus*	*Listeria monocytogenes*
0.01	Turbidity	Turbidity	Turbidity	Turbidity
0.02	Turbidity	Turbidity	Turbidity	Turbidity
0.03	Turbidity	Turbidity	Turbidity	No Turbidity
0.04	No Turbidity	Turbidity	No Turbidity	No Turbidity
0.05	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.06	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.07	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.08	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.09	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.10	No Turbidity	No Turbidity	No Turbidity	No Turbidity

n=6

Optimization of level of oregano essential oil

The MIC of oregano oil was determined by using different concentrations *viz.* 0.01% to 0.1%, against different test bacteria (Table 1, Fig 1,2,3,4). The results indicated that different test bacteria showed different MIC. The MIC of oregano oil against *Escherichia coli*, *Salmonella pullorum*, *Staphylococcus aureus* and *Listeria monocytogenes* were found to be 0.02, 0.03, 0.02 and 0.03%, respectively (Table 3).

Table 3. Minimal inhibitory concentration (MIC) of oregano and thyme essential oil against different test organism

Test organism	Essential oil (%)	
	Oregano	Thyme
Escherichia coli	0.02	0.04
Salmonella pullorum	0.03	0.05
Staphylococcus aureus	0.02	0.04
Listeria monocytogenes	0.03	0.03

Optimization of level of thyme essential oil

The MIC of thyme essential oil was determined by using different concentrations *viz.* 0.01% to 0.1%, against different test bacteria (Table 2, Fig 1,2,3,4). The results indicated that different test bacteria showed different MIC. The MIC of thyme oil against *Escherichia coli*, *Salmonella pullorum*, *Staphylococcus aureus* and *Listeria monocytogenes* were found to be 0.04, 0.05, 0.04 and 0.03%, respectively (Table 3).

Table 4. Different combinations of oregano and thyme essential oil against test bacteria for calculation of minimal inhibitory concentration (MIC)

Essential oil (%) oregano	thyme	*Escherichia coli*	*Salmonella pullorum*	*Staphylococcus aureus*	*Listeria monocytogenes*
0.02	0.03	No Turbidity	No Turbidity	No Turbidity	No Turbidity
0.01	0.03	Turbidity	Turbidity	Turbidity	Turbidity
0.005	0.03	Turbidity	Turbidity	Turbidity	Turbidity
0.02	0.02	Turbidity	No Turbidity	No Turbidity	Turbidity
0.01	0.02	Turbidity	Turbidity	Turbidity	Turbidity
0.005	0.02	Turbidity	Turbidity	Turbidity	Turbidity
0.02	0.01	Turbidity	Turbidity	Turbidity	Turbidity
0.01	0.01	Turbidity	Turbidity	Turbidity	Turbidity
0.005	0.01	Turbidity	Turbidity	Turbidity	Turbidity

n=6

Optimization of level of combined concentration of oregano and thyme essential oils

The different concentrations of oregano and thyme essential oils in combination were used against *Escherichia coli*, *Salmonella pullorum*, *Staphylococcus aureus* and *Listeria monocytogenes* for calculation of MIC (Table 4, Fig 1,2,3,4). The results of MIC of combined level oregano and thyme essential oils against above test bacteria were presented in Table 5. On the basis of above experiments, the final concentration of oregano and thyme essential oils selected for incorporation in edible film as biopreservative were 0.02% and 0.03%, respectively (Table 6).

Table 5. Minimal inhibitory concentration (MIC) of combination of oregano and thyme essential oil against different test organism

Test organism	Essential oil (%)	
	Oregano	Thyme
Escherichia coli	0.02	0.03
Salmonella pullorum	0.02	0.02
Staphylococcus aureus	0.02	0.02
Listeria monocytogenes	0.02	0.03

Table 6. The final concentration of oregano and thyme essential oils used in edible film as biopreservative

Essential oil	Concentration (%)
Oregano	0.02
Thyme	0.03

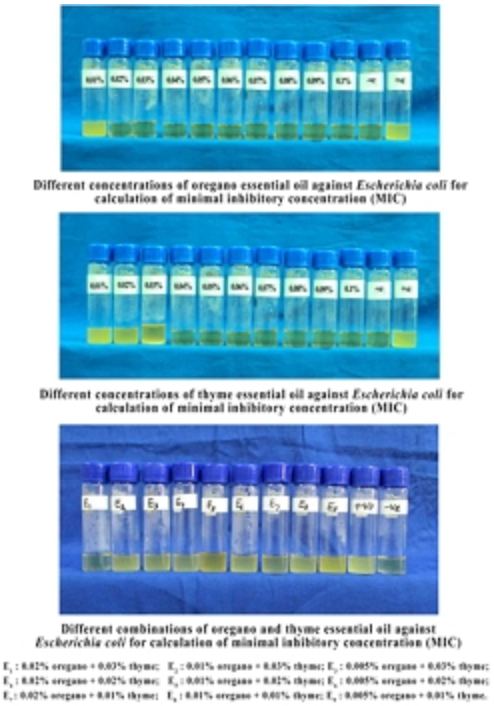

Fig. 1. Determination of minimal inhibitory concentration (MIC) of essential oils against *Escherichia coli* by tube dilution method

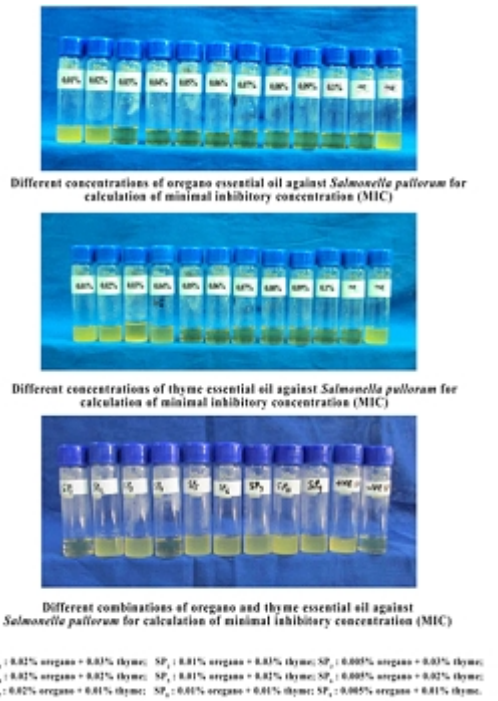

Fig. 2. Determination of minimal inhibitory concentration (MIC) of essential oils against *Salmonella pullorum* by tube dilution method

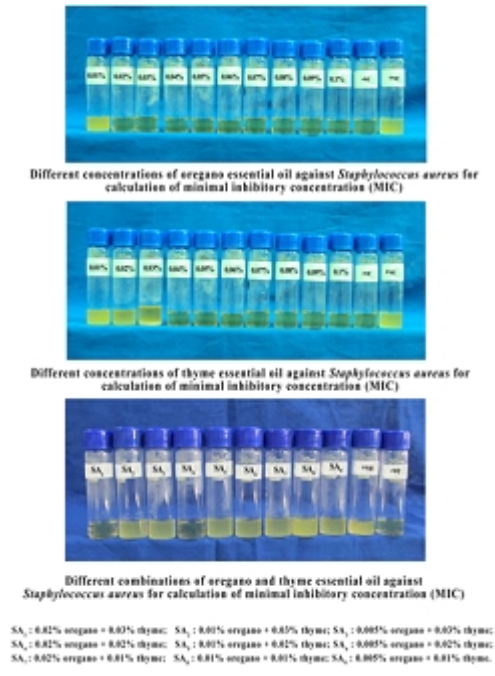

Fig. 3. Determination of minimal inhibitory concentration (MIC) of essential oils against *Staphylococcus aureus* by tube dilution method

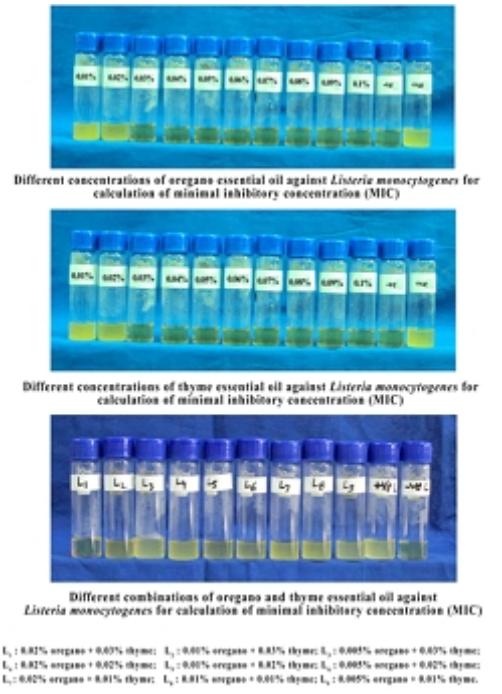

Fig. 4. Determination of minimal inhibitory concentration (MIC) of essential oils against *Listeria monocytogenes* by tube dilution method

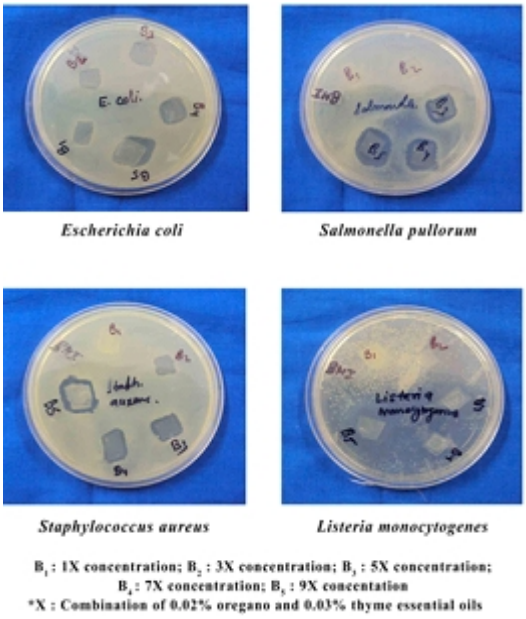

Fig. 5. Disc diffusion test against selected bacteria for efficacy of edible film coated with essential oils

Antimicrobial property of edible film coated with essential oil

Standardized edible film (1.5% carrageenan) coated with standardized combined concentration of oregano and thyme essential oil (Table 6) was evaluated for its antimicrobial property against different test bacteria *viz*. *Escherichia coli, Salmonella pullorum, Staphylococcus aureus* and *Listeria monocytogenes* by disc diffusion test. The results of disc diffusion test showed that no inhibition zone observed at standardized combined concentration of oregano (0.02%) and thyme (0.03%) essential oil. Then, increased concentration of oils was tried *viz.* 3X, 5X, 7X, 9X (X= oregano 0.02% and thyme 0.03%) in edible film and again evaluated for its antimicrobial property (Fig 6). The results showed that 5X concentration produced sufficient inhibition zone against all test bacteria. Therefore 5X concentration (5X =oregano 0.10% and thyme 0.15%) was selected as the final level of essential oils for coating the edible film.

CONCLUSION

The minimal inhibitory concentration assessment by tube dilution method revealed oregano and thyme essential oils at a concentration of 0.02 and 0.03% respectively were effective as antimicrobial against *Escherichia coli, Salmonella pullorum, Staphylococcus aureus* and *Listeria monocytogenes* organisms. The disc diffusion test indicated that a cocktail of oregano and thyme essential oils at the concentration of 0.10% and 0.15%, respectively showed best results against the test bacteria for incorporation in the edible film.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, Indian Veterinary Research Institute, Izatnagar for the facilities provided. The work was carried out using the institute fund provided for the project approved by The Director and The Joint Director (Research), Indian veterinary Research Institute.

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