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Improving Antibacterial Activity of Edible Films Based on Chitosan by Incorporating Thyme and Clove Essential Oils and EDTA

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Abstract: The objectives of this study were to study the antibacterial effect of thyme and clove essential oils as well as their synergistic effect with chelating agent (EDTA) from chitosan-based edible films against gram-positive and gram-negative bacteria. Thyme and clove essential oils were incorporated into chitosan-based edible films at 0.5, 1 and 1.5% v/v both alone and in combination with EDTA at 5 mmol. Films containing thyme essential oil revealed larger inhibitory zones than those of containing clove essential oil. EDTA enhanced antimicrobial effects of essential oils against both gram-positive and gram-negative bacteria. The results of this study showed that thyme and clove essential oils in combination with EDTA have a good potential for using with chitosan to make antimicrobial edible films and coatings for various food applications.

Key words: Active packaging, chelating agent, thymol, carvacrol, eugenol

INTRODUCTION

Microbial growth on food surfaces is a major cause of food spoilage (Siragusa and Dickson, 1992). There have been remarkable developments in recent years in the polymeric and edible packaging films incorporated with antimicrobial agents for improving the preservation of packaged foods (Hoffman *et al*., 2001; Cha *et al*., 2002; Janes *et al*., 2002; Mecitoglu *et al*., 2006). These films possess the potential for improving microbial stability of foods by acting on the food surface, upon contact.

Chitosan β 1,4 linked glucosamine and N-acetyl glucosamine is a polysaccharide prepared by deacetylation of chitin, which is one of the most abundant natural polymers in living organisms such as crustacean, insects and fungi (Coma *et al*., 2002). The structure of chitosan is shown in Fig. 1.

Chitosan is determined as a non-toxic, a biodegradable and a biocompatible polymer. It has antimicrobial properties (Coma *et al*., 2002). Interestingly some antibacterial and antifungal activities have been described with chitosan and modified chitosan with the knowledge that this aminopolysaccharide was shown to reduce microbial growth (Chen *et al*., 1998; Tsai *et al*., 2000; Molloy *et al*., 2004; Pranoto *et al*., 2005a; Sébastien *et al*., 2006). Chitosan-based edible films have good mechanical strength and are excellent oxygen barriers; however, due to their hydrophilic nature, they have poor moisture barrier properties (Miller and Krochta, 1997; Caner *et al*., 1998; García *et al*., 2004). This property can be improved by the addition of hydrophobic and crosslinking materials (Suyatma *et al*., 2004; Möller *et al*., 2004).

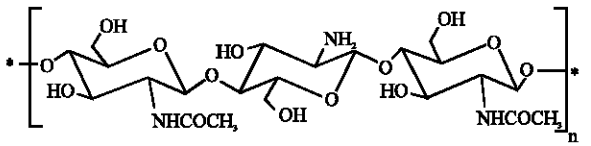

Fig. 1: Structure of chitosan

Many spices and herbs and their extracts possess antimicrobial activity, which minimize questions regarding their safe use in food products (Holley and Patel, 2005). Essential oils and their constituents have wide spectra of antimicrobial action (Packiyasothy and Kyle, 2002). The composition, structure as well as functional groups of the oils play an important role in determining their antimicrobial activity (Holley and Patel, 2005). Usually compounds with phenolic groups are most effective (Dorman and Deans, 2000). Among these, the oils of clove, thyme, cinnamon, rosemary, sage and vanillin have been found to be most consistently effective against microorganisms. They are generally more inhibitory against gram-positive than against gram-negative bacteria (Mangena and Muyima, 1999). Because of the effect of direct addition of essential oils to food on sensory characteristics of added food, incorporation of essential

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oils to edible films may have supplementary applications in food packaging (Pranoto *et al*., 2005a, b; Seydim and Sarikus, 2006).

EDTA, a food grade chelator, can have an antimicrobial effect by limiting the availability of cations and destabilizing the cell membranes of bacteria by complexing divalent cations, which act as salt bridges between membrane macromolecules, such as lipopolysaccharides (Cha *et al*., 2002). Treatments with chelators can alter the outer membrane permeability of gram-negative bacteria. In such cases, gram-negatives showed sensitivity to nisin (Boziaris and Adams, 1999; Grill and Holley, 2000, 2003).

The objectives of this study were to prepare antimicrobial edible films based on chitosan incorporated with thyme and clove essential oils. In order to study the synergistic effect of antimicrobials and chelating agent against bacterial strains, EDTA was incorporated into chitosan-based films.

MATERIALS AND METHODS

Bacterial strains and maintenance: This study was conducted from May till July 2007 at Department of Food Science and Engineering, Faculty of Biosystem Engineering, University of Tehran, Iran. The bacterial strains used in this study were *Listeria monocytogenes* PTCC 1298, *Staphylococcus aureus* PTCC 1431, *Salmonella enteritidis* PTCC 1318 and *Pseudomonas aeroginosa* PTCC 1247. These bacteria were obtained from Persian Type Culture Collection (Tehran, Iran). The bacterial cultures were grown on the nutrient agar slant (Merck Co., Darmstadt, Germany) and kept at 4°C. Subculturing was carried out each 21 days to maintain bacterial viability. Overnight cultures of bacterial strains were grown and agitated at 140-150 rpm in an incubator shaker for 24 h in Brain Heart Infusion (Merck Co., Darmstadt, Germany) at 37°C. A dilution series was carried out to meet required bacterial population for seeding, by using sterile distilled water.

Preparation of antimicrobial edible films: Preparation of antimicrobial edible films was conducted at Food Analysis and Chemistry Laboratory, Department of Food Science and Engineering, Faculty of Biosystem Engineering, University of Tehran, Iran. Chitosan-based edible film was prepared by dissolving practical grade (85% deacetylated) chitosan from crab shells (Sigma Chemical Co., St. Louis, Mo., USA) in an aqueous solution (1% v/v) of glacial acetic acid to a concentration of 2% (w/v) while stirring on a magnetic stirrer/hot plate. The solution was stirred with low heat (at 40°C) which typically required 6 h stirring. The resultant chitosan solution was filtered through a Whatman No. 3 filter paper to remove any dissolved particles. After filtration the solution was returned to the magnetic stirrer/hot plate and Glycerol (Sigma Chemical Co., St. Louis, Mo., USA) was added to a level of 0.50 mL g^{-1} chitosan as a plasticizer. The plasticizer was mixed into the solution for 30 min. Thyme and clove essential oils (obtained from Giah-Essence Co., Gorgan, Iran) in 0.5, 1 and 1.5% ratios were added to chitosan film forming solution as essential oil concentration per film. In order to study the synergistic effect of antimicrobials and chelating agent from chitosan-based films against bacterial strains 5 mmol of EDTA (Sigma Chemical Co., St. Louis, Mo., USA) was added into film forming solutions (Stevens *et al*., 1991). The film forming solutions were degassed under vacuum for 5 min. The film forming solutions (45 mL) were casted on the center of 14.5 cm circular glass plates. The films were dried for 30 h at ambient conditions (25°C). Dried films were peeled and stored in a dessicator at 51% RH and 25°C until evaluation. Saturated magnesium nitrate solution was used to meet required relative humidity.

Determination of antimicrobial effects of edible films: The agar diffusion method was used for determining the antibacterial effects of edible films on bacterial strains. The edible films were cut into 10 mm diameter discs with a circular knife. Film cuts were placed on brain heart infusion agar (BHI Agar). Agar plates had been previously seeded with 0.1 mL of an overnight broth culture of indicator strains. Initial number of bacteria was in the range of 10^5-10^6 cfu mL^{-1}. Bacterial strains were incubated at 37°C for 24 h. The diameter of the zone of inhibition was measured with a caliper to the nearest 0.02 mm. The whole zone area was calculated then subtracted from the film disc area and this difference in area was reported as the zone of inhibition (Seydim and Sarikus, 2006).

Statistical analysis: The triplicate data were performed to an analysis of variance for the significance of added essential oils and EDTA based on the area of inhibition using MSTATC programs. Duncan's Multiple Range Tests was used to compare the difference among means at the level of 0.05.

RESULTS AND DISCUSSION

Effects of antimicrobial agents in chitosan-based films on inhibition zone area against gram-positive and gram-negative bacteria are shown in Table 1. When antimicrobial agents are incorporated, there will be

Table 1: Antibacterial activity (inhibitory zone) of chitosan films incorporated with thyme and clove essential oils and EDTA against gram-positive and gram-negative bacteria

Essential oils	*L. monocytogenes*		*S. aureus*		*S. enteritidis*		*Ps. aeroginosa*	
	Without EDTA	With EDTA	Without EDTA	With EDTA	Without EDTA	With EDTA	Without EDTA	With EDTA
Thyme								
Control	0^{i}_{B}	33.05^{d}_{A}	0^{d}_{B}	42.15^{d}_{A}	0^{d}_{B}	15.10^{d}_{A}	0^{c}_{B}	54.12^{d}_{A}
0.5%	76.16^{e}_{B}	95.17^{c}_{A}	61.43^{c}_{B}	79.34^{c}_{A}	29.18^{c}_{B}	38.16^{c}_{A}	0^{c}_{B}	62.35^{c}_{A}
1.0%	131.14^{b}_{B}	150.05^{b}_{A}	109.03^{b}_{B}	138.17^{b}_{A}	48.12^{b}_{B}	66.74^{b}_{A}	21.43^{b}_{B}	78.41^{b}_{A}
1.5%	220.13^{a}_{B}	250.48^{a}_{A}	162.89^{a}_{B}	174.63^{a}_{A}	75.61^{a}_{B}	101.55^{a}_{A}	35.19^{a}_{B}	92.18^{a}_{A}
Clove								
Control	0^{i}_{B}	33.05^{d}_{A}	0^{d}_{B}	42.15^{d}_{A}	0^{c}_{B}	15.10^{d}_{A}	0^{b}_{B}	54.12^{c}_{A}
0.5%	40.42^{e}_{B}	65.46^{c}_{A}	37.40^{c}_{B}	56.36^{c}_{A}	0^{c}_{B}	24.21^{c}_{A}	0^{b}_{B}	56.18^{c}_{A}
1.0%	85.32^{b}_{B}	113.79^{b}_{A}	65.48^{b}_{B}	92.08^{b}_{A}	28.44^{b}_{B}	56.35^{b}_{A}	0^{b}_{B}	69.47^{b}_{A}
1.5%	133.83^{a}_{B}	157.79^{a}_{A}	98.14^{a}_{B}	110.87^{a}_{A}	53.64^{a}_{B}	79.31^{a}_{A}	13.71^{a}_{B}	81.72^{a}_{A}

Means in each row for each bacterium with different subscript letter(s) are significantly different ($p<0.05$). Means in each column with different superscript letter(s) are significantly different ($p<0.05$). Inhibitory zone is the area of inhibition, which is surrounding film discs. It was calculated by subtracting the whole zone area from the film disc area and measured in mm^2. Control is a film disc containing no essential oil

diffusing materials through agar gel and furthermore, resulting clearing zone on the bacterial growth (Pranoto *et al*., 2005a). Chitosan-based films incorporated with thyme essential oil produced larger inhibition zone than those of incorporated with clove essential oil when the same level of essential oil was present in each film, which is attributed to the varying level of antimicrobial activity.

Incorporation of thyme essential oil at higher than 0.5% v/v exhibited a clear inhibitory zone by the absence of bacterial growth around the film cuts. At thyme oil concentration of 0.5% v/v the clear zone of inhibition was not observed with *Ps. aeroginosa*. As the concentration increased, the zone of inhibition also increased significantly. The antimicrobial activity of thyme has been attributed to their essential oils which contain the terpenes: carvacrol [2-methyl-5-(1-methylethyl) phenol] and thymol [5-methyl-2-(1-methylethyl) phenol] respectively. Cosentino *et al*. (1999) suggested that components of the essential oil may have acted synergistically (e.g., thymol and carvacrol) or antagonistically (e.g., p-cymene) with other components to alter overall antimicrobial effectiveness. According to Burt (2004), carvacrol and thymol disintegrate the outer membrane of gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP. The presence of magnesium chloride has been shown to have no influence on this action, suggesting a mechanism other than chelation of cations in the outer membrane (Helander *et al*., 1995). In one study 2% of oregano essential oil incorporated to whey protein-based edible films was found to be effective against *E. coli* O157:H7, *S. aureus*, *S. enteritidis* and *L. monocytogenes* (Seydim and Sarikus, 2006). Source and concentration of active components of the plant essential oils as well as basic film material have crucial effect on their antimicrobial activity in edible films.

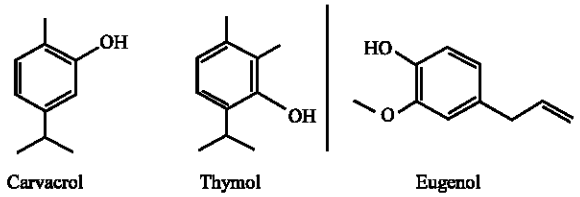

Fig. 2: Structures of the major components of thyme and clove essential oils

Films containing 0.5% clove essential oil were not inhibitory against *S. enteritidis* and *Ps. aeroginosa*. However, they were effective against *L. monocytogenes* and *S. aureus*. Clove essential oil were effective against all bacterial strains at concentration of 1.5%. The main inhibitory component of clove essential oil is believed to be eugenol (Matan *et al*., 2006). Sub-lethal concentrations of eugenol have been found to inhibit production of amylase and proteases by *Bacillus cereus*. Cell wall deterioration and a high degree of cell lysis were also noted (Thoroski *et al*., 1989). The structures of major components of thyme and clove essential oils are shown in Fig. 2.

The antimicrobial agents were obviously more effective against gram-positive bacteria than the gram-negative bacteria tested. This difference is due to the cell wall structures of bacteria so that gram-positive bacteria are more sensitive to such agents (Nychas, 1995). In the gram-positive bacteria, the major constituent of its cell wall is peptidoglycan and very little protein. On the other hand the cell wall of gram-negative bacteria is thinner but more complex and contains various polysaccharides, proteins and lipids beside peptidoglycan (Pranoto *et al*., 2005a). The cell wall of gram-negative bacteria also has outer membrane, which constitutes the outer surface of the wall (Black, 1996). Among the bacteria examined, *Ps. aeroginosa* and *L. monocytogenes* were the most resistant and susceptible to essential oils, respectively.

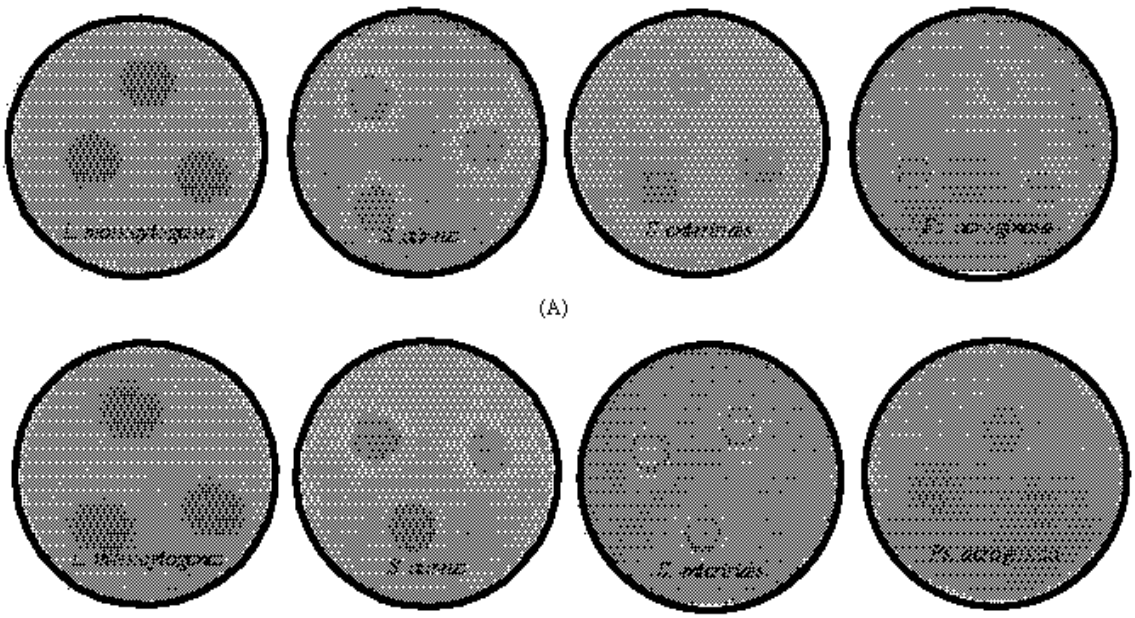

Fig. 3: Representative pictures of inhibitory zones of chitosan films incorporated with 1.5% thyme essential oil against Four test microorganisms (A) films containing no EDTA and (B) films containing EDTA

Same results were reported by Dorman and Deans (2000), Ruberto *et al*. (2000), Senatore *et al*. (2000), Pintore *et al*. (2002), Wilkinson *et al*. (2003) and Cha *et al*. (2003). Although the high resistance of *Ps. aeroginosa* toward essential oils, this bacterium was the most sensitive to EDTA. Nonetheless, since pseudomonads are so frequently responsible for spoilage of food stored at low temperatures they have often been used as targets. Only at high concentration of thyme essential oil this bacterium was inhibited, however films containing thyme and EDTA were obviously more effective. Generally, films containing essential oil and EDTA showed larger inhibitory zones. This is due to the synergistic effect of essential oil and EDTA. Chelating agents such as EDTA bind magnesium ions in the lipopolysaccharide layer and produce cells with increased susceptibility to antibiotics and detergents (Nikaido and Vaara, 1987). Treatment with EDTA is reported to alter the outer membrane permeability of gram-negatives (Vaara, 1992). Grill and Holley (2000) studied the effect of combination treatment of lysozyme, nisin and EDTA ability to control growth of gram-positive and gram-negative bacteria. Sample pictures of inhibitory effect of chitosan films incorporated with 1.5% thyme essential oil against four test microorganisms are shown in Fig. 3.

Chitosan-based control films (without EDTA) did not show inhibitory zone in bacterial strains tested. Despite antimicrobial activity of chitosan because of its innate characteristic, this effect of chitosan occurred without migration of active agents. Chitosan does not diffuse through the adjacent agar media; so that only organisms in direct contact with the active sites of chitosan are inhibited.

CONCLUSION

Antimicrobial property of chitosan-based films enriched with essential oils was shown in this study. Because of the effect of direct addition of essential oils to food on sensory characteristics of added food, incorporation of essential oils to edible films may have supplementary applications in food packaging. This study indicates that the addition of thyme and clove essential oils and EDTA has the potential of application in antimicrobial packaging, both in gram-negative and gram-positive bacteria contaminating foods.

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