Evaluation of the antibacterial and antioxidant activities of chitosan edible films incorporated with organic essential oils obtained from four *Thymus* species

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Abstract The aims of this study were to evaluate, (1) the antibacterial properties, (2) the total phenol content and (3) the antioxidant activity, of chitosan edible films incorporated with certified organic essential oils (EOs) obtained from *Thymus zygis*, *Thymus mastichina*, *Thymus capitatus* and *Thymus vulgaris*. The agar disc diffusion method was used to determine the antibacterial activities of chitosan edible films while for the antioxidant activity, two different analytical assays were used (DPPH and FRAP). As regard antibacterial activity, films containing only chitosan were not effective against any of tested bacteria. Chitosan films containing *T. capitatus* EO (CH + TCEO) was more effective against *Listeria innocua* and *Alcaligenes faecalis* whilst chitosan films containing *T. mastichina* EO (CH + TMEO) had the highest inhibition halos against *Serratia marcescens*. For and *Enterobacter amnigenus* no antibacterial activity was achieved. Chitosan films added with *Thymus* essential oils showed antioxidant activity, at all concentrations and with all methods assayed. CH + TCEO had the highest antioxidant activity revealed with DPPH assay. However in CH + TCEO showed best antioxidant effect when assessed with FRAP assay. The results showed that chitosan edible films incorporated with organic *Thymus* EOs could be used as active films in food industry due to its antibacterial and antioxidant activities.

Keywords Essential oils · Chitosan films · *Thymus* · Antibacterial · Antioxidant

Introduction

At present, the amount of research involving the production and characterization of biodegradable films has increased substantially, mainly due to interest in minimizing the ecological impact caused by the use of synthetic packaging materials. Furthermore, biodegradable films are excellent vehicles for incorporating a wide variety of additives, such as antioxidants, antifungal and antimicrobials agents as well as dyes (Rhim and Ng 2007). Chitosan, poly- β -(1/4) *N*-acetyl-D-glucosamine, has a great potential to be used as alternative resources for active food packaging or food applications due to its biodegradability, biocompatibility, non-toxicity and film-forming capacity (Tharanathan and Kittur 2003). In addition, this substance has intrinsic antioxidant and antimicrobial properties, which are affected by its molecular weight and concentration. Therefore, chitosan has been used as antimicrobial films and coatings due to its effectiveness of inhibiting the growth of Gram-positive and Gram-negative bacteria as well as yeasts and moulds (Siripatrawan and Harte 2010). Additionally, functional substances, such as polyphenolic compounds or essential oils have been incorporated into chitosan to enhance its antibacterial and antioxidant activity (Ruiz-Navajas et al. 2013; Sánchez-González et al. 2010). Essential oils (EOs) are gaining interest for their potential as preservatives and as decontamination agents since such substances have been generally recognized as safe (GRAS) and they are widely accepted by consumers (Burt 2004). Nevertheless, direct application to foods, as a preservative, may be limited, since (1) it can be lost during storage due to its high volatility (2) it can react

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with various food components and (3) it can have detrimental effects on the organoleptic properties. Nonetheless, the EOs can be added to edible films and maintain all its properties. Thus, the aims of this study were to evaluate (1) the antibacterial properties, (2) the total phenol content (TPC) and (3) the antioxidant activity, of chitosan edible films incorporated with certified organic EOs of *Thymus zygis*, *Thymus mastichina*, *Thymus capitatus* and *Thymus vulgaris*.

Materials and methods

Essential oils

The essential oils (EOs) of *Thymus zygis* ref. 11961 (TZEO), *Thymus mastichina* ref. 90001–1284 (TMEO), *Thymus capitatus* ref. 95001–1150 (TCEO), and *Thymus vulgaris* ref. 80001–3577 (TVEO) were used in this work. These EOs were analysed by Ballester-Costa et al. (2013). These authors reported that in TMEO the major compounds were 1,8-cineole (51.94 %), and linalool (19.90 %). TCEO was characterized by the presence of carvacrol (69.83 %), and their precursors *p*-cymene (6.12 %) and γ -terpinene (6.68 %). As regards to TVEO, the main component was linalool (44.00 %) followed by terpineol-4 (11.84 %). Finally, in TZEO the major components were thymol (48.59 %) and *p*-cymene (18.79 %). All EOs analysed were supplied by Esencias Martinez Lozano (Murcia, Spain). The EOs were organic certified by the Institute for Marketecology (IMO) according to the procedures as outlined in the USDA, AMS 7 CFR Part 205 National Organic Program, Final Rule.

Preparation of edible films

Chitosan-based film was prepared by dissolving chitosan of high molecular weight and 75–85 % deacetylated (Sigma-Aldrich Chemical Co., Steinheim, Germany) in a lactic acid aqueous solution (1 % v/v) at a concentration of 2 % (w/v) following the recommendations of Ojagh et al. (2010). The nine samples obtained were: Chitosan film (CH), chitosan added with TZEO at 1 and 2 % (CH + TZEO 1 and CH + TZEO 2 %), chitosan added with TMEO at 1 and 2 % (CH + TMEO 1 and CH + TMEO 2 %), chitosan added with TVEO at 1 and 2 % (CH + TVEO 1 and CH + TVEO 2 %) and chitosan added with TCEO at 1 and 2 % (CH + TCEO 1 and CH + TCEO 2 %).

Antibacterial activity

Microbial strains

The chitosan films incorporated with different *Thymus* EOs were individually tested against *Listeria innocua* CECT 910,

Serratia marcescens CECT 854, *Enterobacter amnigenus* CECT 4078 and *Alcaligenes faecalis* CECT 145. These bacteria were chosen based on they are commonly isolated from refrigerated foods (mainly meat and meat products) or as indicators or models of food pathogenic bacteria. All strains were supplied by the Spanish Type Culture Collection (CECT) of the University of Valencia (Spain).

Antibacterial assay

The agar disc diffusion method described by Tepe et al. (2005) with some modifications was used to determine the antibacterial capacity of chitosan edible films incorporated with TZEO, TMEO, TVEO or TCEO. Briefly, a suspension (0.1 mL of 10^6 cfu/mL) of each microorganism was spread on the solid medium plates; nutrient Agar II in the case of *S. marcescens*, *E. amnigenus* and *A. faecalis*; and Brain Heart Infusion agar for *L. innocua*. CH, CH + TZEO, CH + TMEO, CH + TVEO or CH + TCEO edible films discs, 10 mm in diameter, were aseptically obtained and placed on the inoculated plates; these plates were incubated at 37 °C for 24 h in the case of *A. faecalis*, *E. amnigenus* and *L. innocua* and at 26 °C for 24 h for *S. marcescens*. The diameters of the inhibition zones were measured in millimetres. All tests were performed in triplicate.

Preparation of extracts for antioxidant activity

Each film sample (1 g) was weighed into a tube test and 30 mL of methanol were added. The mixture was vigorously shaken for 2 min and left for 2 h in an ultrasonic water bath with-out temperature control. Then, the tube was then centrifuged at $2739 \times g$ for 20 min at 4 °C and the supernatant was used to determine the total phenol content and the antioxidant properties.

Total phenol content

The total phenol content (TPC) was determined using the Folin-Ciocalteu reagent (Singleton and Rossi 1965). The results were expressed in mg gallic acid equivalents (GAE)/g films, as mean of three replicates.

Antioxidant activity

DPPH radical scavenging assay

The efficacy of the films to scavenge 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radicals was determined using the methodology described by Siripatrawan and Harte (2010). Results were expressed as μg Trolox equivalent/g film as mean of three replicates.

Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) of the different films samples was determined by using the potassium ferricyanide-ferric chloride method (Oyaizu 1986). Results were expressed as mg Trolox equivalent/g film as mean of three replicates.

Statistical analysis

Data collected for antioxidant and antibacterial properties were analysed by two-way analysis of variance to test the effects of two fixed factors: concentration (levels: 1 and 2 %) and EO (levels: *T. zygis*, *T. capitatus*, *T. mastichina* and *T. vulgaris*). The Tukey post hoc test was applied for comparisons of means, differences were considered significant at $p > 0.05$. Statistical analysis and comparisons among means were carried out using the statistical package Statgraphics 5.1 for Windows.

Results and discussion

Antibacterial activity

Inhibition zone diameters yielded by chitosan edible film disks with various concentrations (0, 1 and 2 %) of *T. zygis*, *T. capitatus*, *T. mastichina* and *T. vulgaris* EOs against tested bacteria are shown in Table 1. Films containing only chitosan were not effective against any of the four tested bacteria. These results were in concordance with Ruiz-Navajas et al. (2013) and Wang et al. (2011) who reported that no significant inhibition zone was observed for the pure chitosan film against *L. innocua*, *S. marcescens*, *Escherichia coli* and *Staphylococcus aureus*.

The antibacterial inhibition activity against *S. marcescens* occurred in a concentration dependent manner. The highest inhibition halos ($p < 0.05$) were obtained for CH + TMEO 2 % followed by CH + TVEO 2 %. CH + TZEO at 1 % was not active against this strain while at 2 % showed the lowest ($p < 0.05$) inhibition halos. As regards *L. innocua*, again the antibacterial activity occurred in a concentration dependent manner. However, CH + TCEO 2 % showed the highest ($p < 0.05$) inhibition halos, followed by CH + TMEO 2 %. Again, CH + TZEO at 1 % was not active against this strain while at 2 % showed the lowest ($p < 0.05$) inhibition halos. With reference to *A. faecalis*, all chitosan films added with *Thymus* EOs, at all concentrations-assayed, showed inhibitory effects which was concentration-dependent. CH + TCEO at 2 % showed the highest ($p < 0.05$) inhibition halos followed, by CH + TZEO at

2 %. CH + TVEO showed the lowest ($p < 0.05$) inhibition halos for this bacteria. *E. amnigenus* was the most resistant strain since the films added with *Thymus* EOs, at all concentrations assayed, did not show any inhibitory effect.

The antibacterial activity of chitosan edible films added with EOs from conventional *Thymus* species has been widely studied. Hosseini et al. (2008) analysed the antibacterial effects of thymes EO incorporated into chitosan-based edible films at 0.5, 1 and 1.5 %. These authors reported that chitosan-based films incorporated with thyme EO produced high inhibition zones against *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Pseudomonas aeruginosa*. Gómez-Estaca et al. (2010) analysed the antimicrobial chitosan films prepared with *Thymus vulgaris* EO. These authors concluded that chitosan-essential oil composite films, showed a significant antimicrobial activity against *Photobacterium phosphoreum* and *Clostridium perfringens* and a moderate activity on *Staphylococcus aureus* and *Bacillus cereus*. In other study, Ruiz-Navajas et al. (2013) reported that the addition of *Thymus piperella* and *Thymus moroderi* EOs to chitosan films, in general, inhibited the growth of *L. innocua*, *Aeromonas hydrophila* and *Achromobacter denitrificans*.

The antibacterial activity of chitosan films added with different *Thymus* EOs could be attributed to action of carvacrol or thymol. In vitro studies had been demonstrated that these compounds exhibit antimicrobial activity against a broad spectrum of both Gram-negative and Gram-positive bacteria (Burt and Reinders 2003; Gaysinsky et al. 2005). However, the action had been attributed to a synergistic effect between various major and minor components. The mechanisms behind the antimicrobial activity are not known. Nevertheless, Viuda-Martos et al. (2011) reported that the reasons behind this may be an attack on the cell membrane's phospholipid bilayer, the disruption of the enzymatic systems, the compromising of the genetic material of the bacteria/yeast, the formation of fatty acid hydroperoxidase by the oxygenation of unsaturated fatty acids, the coagulation of the cytoplasm, the damage of lipids and proteins, the distortion of the proton motive force (PMF), the electron flow and/or the active transport. Burt (2004) mentioned that EOs could disintegrate the outer membrane of bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to adenosine tri-phosphate.

Total phenolic content and Antioxidant activity

Total phenolic content (TPC) of chitosan edible films incorporated with TZEO TCEO, TVEO and TMEO, at different concentrations, is shown in Fig. 1. Chitosan films (control sample) showed a TPC of 0.27 mg GAE/g film.

Table 1 Antibacterial effect of chitosan edible films added with organic *Thymus zygis* (CH + TZEO), *Thymus capitatus* (CH + TCEO), *Thymus vulgaris* (CH + TVEO) and *Thymus mastichina* (CH + TMEO) EOs against Gram-positive or Gram-negative bacteria

Films	Diameter of inhibition zone (mm) including film (10 mm)			
	<i>Serratia marcescens</i>	<i>Listeria innocua</i>	<i>Alcaligenes faecalis</i>	<i>Enterobacter amnigenus</i>
Control	N.A.	N.A.	N.A.	N.A.
CH + TZEO 1 %	N.A.	N.A.	28.41 ± 0.03 ^C	N.A.
CH + TZEO 2 %	12.50 ± 0.37 ^{cG}	16.35 ± 0.70 ^{bE}	32.12 ± 0.83 ^{aB}	N.A.
CH + TCEO 1 %	13.19 ± 0.24 ^{cF}	18.28 ± 0.18 ^{bC}	31.66 ± 0.29 ^{aB}	N.A.
CH + TCEO 2 %	19.90 ± 0.14 ^{cD}	27.36 ± 0.78 ^{bA}	35.08 ± 1.20 ^{aA}	N.A.
CH + TVEO 1 %	18.49 ± 0.05 ^{aE}	12.19 ± 0.06 ^{cF}	14.35 ± 0.11 ^{bF}	N.A.
CH + TVEO 2 %	26.49 ± 0.08 ^{aB}	16.49 ± 0.06 ^{cE}	21.23 ± 0.18 ^{bD}	N.A.
CH + TMEO 1 %	21.15 ± 0.04 ^{aC}	17.92 ± 0.08 ^{cD}	18.42 ± 0.12 ^{bE}	N.A.
CH + TMEO 2 %	32.36 ± 0.05 ^{aA}	25.51 ± 0.04 ^{cB}	28.29 ± 0.04 ^{bC}	N.A.

N. A. non active

For a same essential oil, values followed by the same lower case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test

For a same bacteria, values followed by the same upper case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test

This was in agreement with the results reported by Moradi et al. (2012) and Ruiz-Navajas et al. (2013) where a low TPC for chitosan film was observed. This finding could be explained to the formation of chromogens, due to the reaction of Folin and Ciocalteu reagent with non-phenolic reducing as mentioned by Moradi et al. (2012). The results showed that the TPC in the chitosan edible films significantly increased ($p < 0.05$) with increase in essential oil concentration. At high concentration (2 %); CH + TZEO showed the highest ($p < 0.05$) TPC of all samples analysed followed by CH + TCEO. However, at the lowest concentration (1 %), no statistical differences were found ($p > 0.05$) between CH + TZEO and CH + TCEO. CH + TVEO had the lowest ($p < 0.05$) TPC.

TPC of CH + TZEO at 1 % and 2 % levels content as was 9.86 and 11.28 mg/g, respectively. In CH + TCEO at 1 % and 2 % levels showed TPC of 9.70 and 10.77 mg GAE/g.

As regard to the antioxidant activity, DPPH radical scavenging activity and reducing power were used to indicate the antioxidant activity of the films. The addition of EOs onto chitosan films enhanced their antioxidant properties compared to the control films and this enhancement was dependent on the type of essential oil used. In the DPPH assay (Table 2), the results showed that the radical scavenging activity of chitosan films was significantly increased ($p < 0.05$) with the EO concentration. The chitosan films without EO (control sample) showed a slight scavenging activity on DPPH. At high concentration assayed (2 %) the CH + TZEO films showed the highest ($p < 0.05$) values (0.55 mg TE/g) followed by

Fig. 1 Total phenolic content of chitosan edible films incorporated with *Thymus zygis* (TZEO), *Thymus capitatus* (TCEO), *Thymus vulgaris* (TVEO) and *Thymus mastichina* (TMEO) essential oils

CH + TCEO whilst CH + TVEO showed the lowest ($p < 0.05$) values. At low concentration (1 %), no statistical differences were found ($p > 0.05$) between CH + TZEO and CH + TCEO and between CH + TCEO and CH + TMEO. Again CH + TVEO showed the lowest ($p < 0.05$) values.

FRAP assay (Table 2) showed that the variation in, antioxidant capacity was concentration dependent manner. As occur in the case of DPPH assay, control showed a slight ability to reduce ferric to ferrous iron (0.08 mg TE/g film). In this assay, at high concentration (2 %), CH + TCEO films had the highest ($p < 0.05$) ability to reduce ferric to ferrous ion, followed by CH + TMEO films while CH + TVEO showed the lowest ability ($p < 0.05$). However, at low concentration (1 %), no statistical differences were found ($p > 0.05$) between

Table 2 Antioxidant effect of chitosan edible films incorporated with organic *Thymus zygis* (CH + TZEO) *Thymus capitatus* (CH + TCEO), *Thymus vulgaris* (CH + TVEO) and *Thymus mastichina* (CH + TMEO) EOs, at different concentrations by means of two different (DPPH and FRAP) antioxidant test

	DPPH (mg TE/g)	FRAC (mg TE/g)
Control	0.01 ± 0.00 ^H	0.08 ± 0.01 ^H
CH + TZEO 1 %	0.35 ± 0.02 ^{bE}	2.13 ± 0.10 ^{bEF}
CH + TZEO 2 %	0.55 ± 0.01 ^{aA}	3.20 ± 0.05 ^{aC}
CH + TCEO 1 %	0.31 ± 0.01 ^{bEF}	2.06 ± 0.07 ^{bF}
CH + TCEO 2 %	0.48 ± 0.01 ^{aB}	4.70 ± 0.10 ^{aA}
CH + TVEO 1 %	0.26 ± 0.01 ^{bG}	1.98 ± 0.03 ^{bG}
CH + TVEO 2 %	0.39 ± 0.01 ^{aD}	2.95 ± 0.02 ^{aD}
CH + TMEO 1 %	0.29 ± 0.01 ^{bF}	2.21 ± 0.01 ^{bE}
CH + TMEO 2 %	0.44 ± 0.01 ^{aC}	3.99 ± 0.01 ^{aB}

Values expressed as mg Trolox Equivalent (TE)/g film

For a same essential oil, values followed by the same lower case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test

For a same antioxidant assay, values followed by the same upper case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test

CH + TCEO and CH + TZEO films as well as between CH + TZEO and CH + TMEO films. CH + TVEO showed the lowest ($p < 0.05$) ability to reduce ferric to ferrous ion.

The antioxidant as well as the antibacterial properties exhibited by chitosan films added with *Thymus* EOs could be related with the presence, of bioactive compounds such as phenolics acids or terpenoids coming from the EOs (Ruiz-Navajas et al. 2013). Thus, compounds present EOs were showed antioxidant activities may be due to their redox properties exerted by various possible mechanisms: free-radical scavenging activity, hydrogen donors, transition-metal-chelating activity, and/or singlet-oxygen-quenching capacity (Liyana-Pathirana and Shahidi 2006). In addition, when the added ingredients exhibited antioxidant properties, the action of such films involved two different mechanisms: the oxygen-barrier effect-reducing the oxygen availability in the product-and the specific activity of the incorporated antioxidant agents (Bonilla et al. 2012).

The antioxidant activity may be correlated with the TPC. Several studies have indicated the relationships between phenolic content and antioxidant activity; Viuda-Martos et al. 2010; Wang et al. 2016. In this work significant correlation between TPC and antioxidant capacity of chitosan edible films incorporated with *Thymus* EOs (FRAP and DPPH values) was obtained. The correlations of TPC-DPPH and TPC-FRAP with TPC were $r = 0.945$ and $r = 0.832$, respectively.

Conclusion

The results showed that chitosan edible films incorporated with organic *Thymus zygis*, *Thymus mastichina*, *Thymus vulgaris* and *Thymus capitatus* essential oils may have additional applications in food packaging to delay microbial growth and to improve the oxidative stability of foodstuffs due to its excellent antibacterial and antioxidant activities.

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