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Fortification of edible zein films with thyme, laurel and orange essential oils and determination of their antifungal and radical scavenging properties

Anreicherung von essbaren Zeinfilmen mit ätherischen Ölen aus Thymian, Lorbeer und Orange und Bestimmung ihrer antifungalen und radikalfangenden Eigenschaften

Cigdem Mecitoglu Gucbilmez[1,2]), Omer Oksuz[1]), Muhammet Arici[3])

Summary

The purposes of this research were: (a) to produce zein films by adding certain essential oils (EOs), (b) to identify their antifungal activity on some yeasts and molds, and (c) to develop an edible zein film that can inhibit the growth of *Aspergillus fumigatus* which was identified in a previous study as the most heat resistant mold isolated from margarines. Zein films fortified with thyme *(Thymus vulgaris),* laurel *(Laurus nobilis)* and orange *(Citrus sinensis)* EO were tested for their antifungal activities against 3 molds and 5 yeasts by measuring inhibition zone diameters. Considering the single effect, thyme EO was determined as the most effective one. Furthermore, the antifungal activities of thyme EO combined with the other EOs were also studied. From the obtained data, films containing thyme EO in combination with laurel EO were found as the most effective against *Aspergillus fumigatus.* Then, minimally inhibitory concentration against *Aspergillus fumigatus* was determined for the zein film obtained from film solutions including only thyme EO as 1.7 % (v/w), combinations of thyme-laurel EO as 1.6 % (v/w). Also, thyme oil was found as EO having the highest radical scavenging activity and total phenolic compounds among the three EOs.

Keywords: Antifungal activity, Radical scavenging activity, Essential oil, Zein film, *Thymus vulgaris*

Zusammenfassung

Die Ziele dieser Forschungsarbeit waren: a) Zeinschichten mit Zugabe bestimmter ätherischer Öle herzustellen, b) ihre antimykotische Aktivität auf einige Hefen und Schimmelpilzen zu bestimmen, c) eine essbare Zeinschicht zu entwickeln, die das Wachstum von *Aspergillus fumigatus* hemmt, welches in einer früheren Studie aus Margarine als hitzebeständigste Form isolierte wurde. Zeinschichten, die mit den ätherischen Ölen von Thymian *(Thymus vulgaris),* Lorbeer *(Laurus nobilis)* und Orange *(Citrus sinensis)* angereichert waren, wurden auf ihre antimykotische Wirkung gegen drei Schimmelpilze und fünf Hefen, mittels Hemmzonenbestimmung, getestet. In Anbetracht des Einzeleffekts wurde Thymian als das wirksamste ätherische Öl bestimmt. Weitere Untersuchungen zur antimykotischen Aktivitäten von Thymian Öl in Kombination mit anderen ätherischen Ölen wurden ebenfalls durchgeführt. Die Daten zeigten, dass Zeinschichten aus Kombinationen von Thymian und Lorbeer Öl am wirksamsten gegen Aspergillus fumigatus waren. Desweiteren wurde eine minimal inhibierende Konzentration gegen Aspergillus fumigatus bestimmt, die aus Thymian Öl in einer Konzentration von nur 1,7% (v / w) bzw. Thymian/Lorbeer Öl in einer Konzentration von 1,6% (v / w) bestand. Unter den drei ätherischen Ölen war Thymian das mit der höchsten radikalfänger Aktivität und mit den meisten phenolischen Verbindungen.

Schlüsselwörter: Antimykotische Aktivität, Radikalfängeraktivität, Ätherisches Öl, Zein Film, *Thymus vulgaris*

Introduction

Minimally processed foods with long shelf life but with less unnatural additives have been demanded by consumers for years (Souza et al., 2007). By using natural antimicrobial compounds, the presence of pathogenic microorganisms in food can be controlled and by this way processed foods with long shelf life can be obtained (Oussalah et al., 2007). Phenolic compounds such as flavonoids and phenolic acids are found very much in spices. Moreover, these compounds have been indicated to possess antioxidant and antimicrobial properties (Oussalah et al., 2007; Coma, 2008; Suppakul et al., 2003). EOs are the secondary metabolites of plants that can be used as antimicrobial agents for the control of food pathogens because of their antimicrobial activities (Yuan and Chen, 2016). Also, US Food and Drug Administration categorize EOs and their constituents as GRAS (Generally Recognized as Safe) (Manso et al., 2013; Persico et al., 2009). That's why EOs have been generally suggested and used as antifungal, antimicrobial (Appendini and Hotchkiss, 2002) and antioxidant agents (Rodriguez-Lafuente et al., 2009) for food products. When EOs are added to foods such as meat products, beside instant decline of bacterial population, changes in sensory properties of food might occur. So the use of EOs in edible films may gain interest (Oussalah et al., 2007). Due to the deterioration caused by fungi in foods, the loss of quality has been increasing (Soliman and Badeaa, 2002). Because of molds' complex structure, inhibition of them is not a simple process. Aspergillus is one of the most common mold genera producing a strong carcinogen, aflatoxin (Fisher and Dott, 2003). There are also important food spoilage yeasts that can be exemplified as *Candida, Pichia, Rhodotorula, Torulopsis, Saccharomyces, Zygossacharomyces, Hansenula* and *Trichosporon* (Souza et al., 2007; Wojtatowicz et al., 2001).

Since consumers prefer tasty, safe food products with a long shelf-life, good quality and a minimal use of preservatives, the packaging technologies for preservation of foods have been improved (Dainelli et al., 2008). Active packaging can be defined as "a kind of packaging in which the package, the product and its environment interact to increase shelf-life or develop safety or sensory properties while keeping the quality of the food" (Suppakul et al., 2003; Quintavalla and Vicini, 2002). Antimicrobial packaging is a type of active packaging (Quintavalla and Vicini, 2002; Han, 2000). Exhibition of antifungal activity is not enough for the design of active packaging material. We also have to take into consideration the compounds giving the property of antifungal activity and optimum concentration of this compound in active packaging material providing maximum inhibition (Manso et al., 2013).

Like microbial spoilage, oxidation is another important problem which affects food quality adversely. Because of this, packaging materials containing antioxidants have been gained popularity as well as packaging materials including antimicrobial agents (Mecitoğlu Güçbilmez, 2007). On the other hand consumers avoid synthetic antioxidants' toxicity risk, so there has been increasing interest to the use of packaging materials containing natural antioxidants such as phenolic compounds (Roman et al., 2016), vitamins E and C instead of synthetic ones (Vermeiren et al., 1999).

The aims of this study were to determine antifungal activities of thyme, laurel and orange EOs and to investigate their usability as substitutes for fungicides to partially or completely inhibit them. By this way we aimed to obtain an alternative packaging material. Additionally, the capacity of EOs in terms of prevention of the oxidative rancidity in fats was also investigated.

Material and Methods

Material and microorganisms

Corn zein, glycerol, gallic acid, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Co. ABD). Dichloran rose bengal chloramphenicol agar (DRBC), Potato Dextrose Agar (PDA), Folin-Ciocalteu, methanol and ethanol were obtained from MERCK KGaA (Germany).

Aspergillus parasiticus NRRL 465, *Aspergillus fumigatus, Paecilomyces variotii* were supplied by the culture collection of the Department of Food Engineering, Namık Kemal University in Tekirdag, Turkey. *Candida kefyr, Candida zeylanoides* 1, *Candida zeylanoides* 2, *Candida lambica* and *Candida sake* were obtained from the Department of Food Engineering, Erciyes University in Kayseri, Turkey.

The EOs from thyme *(Thymus vulgaris),* laurel *(Laurus nobilis)* and orange *(Citrus sinensis)* were provided by Talya Bitkisel Ürünler Tic. ve San. Ltd. Şti. in Antalya, Turkey.

Preparation of zein film

Zein films were produced according to Padgett et al. (1998). 1.4 g corn zein was dissolved in 8.1 mL of ethanol for 25 minute by mixing slowly. 0.39 mL glycerol was added to the mixture and temperature was increased until it began to boil. Mixing process was then stopped and the mixture was boiled for 5 minute. After cooling the film solution at room temperature, EOs were added at different concentrations and the mixture was again mixed. 4.82 g of zein film solution was poured to the 9x9 cm glass plate which was already cleaned with ethanol. After drying the films at room temperature for 24 hours, they were peeled from glass plate (Fig. 1). The thickness of zein films was determined by taking 10 measurements along random positions of films with a digital micrometer. Average thickness of zein films were determined between 0.100–0.139 mm for pure zein films, thyme EO incorporated zein films, laurel EO incorporated zein films, citrus EO incorporated zein films, laurel and thyme EOs incorporated zein films and potassium sorbate incorporated zein films.

Cultivation of mold and yeast cultures

All cultures of mold and yeast were grown on PDA at 30 °C for 4 days before every use. The renewed cultures were stored at +4 °C.

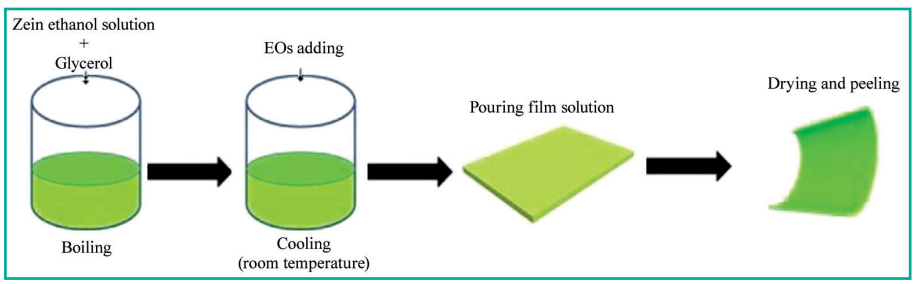

FIGURE 1: *Preparation of zein films with EOs.*

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Determination of the antifungal activity of zein films

Antifungal activity of the films was evaluated with the disc diffusion method. The dilutions were adjusted according to 0.5 Mc Farland standard approx. (10^6 CFU/mL) and prepared from the grown mold and yeast on PDA. 0.1 mL of the standard was spread on the DRBC agar. For antifungal tests, discs were prepared from zein films that were prepared from film solutions containing 3%, 3.5%, 4%, 5%, 6% (v/w) concentrations of thyme EO, 3%, 4%, 5%, 7%, 8% (v/w) concentrations of laurel EO, 3%, 4%, 5%, 6%, 8% (v/w) concentrations of orange EO by a 1.1 cm diameter cork borer under aseptic conditions. Also for control, discs from zein film without EOs were used. The discs were placed on the petri dishes containing DRBC agar inoculated with *Aspergillus parasiticus* NRRL 465, *Aspergillus fumigatus* and *Paecilomyces variotii, Candida kefyr, Candida zeylanoides* 1, *Candida zeylanoides* 2, *Candida lambica, Candida sake* and incubated at 25 °C for 3 days.

After that for determination of minimum inhibition concentration of zein film on *Aspergillus fumigatus,* the discs prepared from zein films including EOs and potassium sorbate were used. The zein film discs that were obtained from film solutions containing 1%, 1.5%, 1.6%, 1.7%, 1.85%, 2%, 2.5% (v/w) concentrations of thyme EO (average amount in discs were between $0.5x10^{-3}$–$1.6x10^{-3}$ mL) and 1.6%, 2.5%, 3.5% (w/w) concentrations of potassium sorbate (average amount in discs were between $0.9x10^{-3}$–$2.3x10^{-3}$ g) were tested on *Aspergillus fumigatus.* At the end of the incubation period, the diameters of the clear inhibition zones seen around the discs were measured with a caliper and the average areas of the zones were calculated.

Also for the antimicrobial tests of zein film discs, including combination of thyme EO with laurel EO and/or orange EO, film discs were prepared from film solutions containing concentrations of 3% (v/w) thyme and 3–4% (v/w) laurel EO (average amount in discs were respectively $2.5x10^{-3}$–$3.4x10^{-3}$ mL), 3% (v/w) thyme and 3% (v/w) orange EO together (average amount in discs was $3.6x10^{-3}$mL), 3% (v/w) thyme, 1.5% (v/w) laurel and 1.5% (v/w) orange EO together (average amount in discs was $3.9x10^{-3}$ mL). The procedures described above were applied to the same molds and yeasts. Afterwards antifungal activities of discs obtained from zein film containing concentrations of 1.5%, 1.6%, 1.65%, 1.7 % (v/w) thyme EO and 1.5%, 1.6%, 1.65%, 1.7 % (v/w) laurel EO (average amount in discs were between $1.7x10^{-3}$–$1.9x10^{-3}$ mL) were tested against *Aspergillus fumigatus* (Fig. 2).

DPPH radical-scavenging activity

DPPH radical-scavenging activity of the EOs was determined according to the spectrophotometry method described by Blois (1958) with minor changes. This method is based on the discoloration of the purple color of DPPH solution prepared in methanol. One mL of various dilutions of EOs at concentrations ranged between 0.752 g/L and 7.52 g/L for thyme, 8.979 g/L and 89.79 g/L for laurel, 84.81 g/L and 848.1 g/L for orange was mixed with 0.1 mM 4 mL freshly prepared DPPH solution in methanol. After 30 min incubation of the mixture in darkness at room conditions, the absorbance of the samples were measured at 517 nm using spectrophotometer (Shimadzu UV Mini-1240, Japan). Three replicates were carried out for each sample. Gallic acid was used as positive control at concentrations varied between 1.25 and 6.25 mg/L. Methanol was used instead of EOs to prepare control sample.

The radical scavenging activities of samples were calculated as given below and expressed as percentage inhibition of DPPH;

$$\text{DPPH radical scavenging (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of control and A_{sample} is absorbance of sample (EOs).

IC_{50} is the value of concentration of EO or gallic acid providing 50% inhibition of DPPH and was calculated from the graph plotted inhibition percentage against EOs or gallic acid concentration.

Determination of amount of total phenolic compounds

Total phenolic compounds were measured by the methods described by Özkan et al. (2007) using gallic acid as a standard phenolic compound. Thyme EO was diluted 10 fold with distilled water. Laurel and citrus EOs were not diluted. 10 µL diluted thyme EO, 50 µL laurel EO and 20 µL citrus EOs were added to a 100 mL volumetric flask containing 75 mL distilled water. 5 mL Folin-Ciocalteu reagent was added and mixed thoroughly. After waiting 3 minutes, 10 mL of saturated sodium carbonate was mixed and completed to 100 mL with distilled water. At the end of 1 hour, absorbance was measured at 760 nm using spectrophotometer. Four replicates were carried out for each sample. Total phenolics in EOs were expressed as gallic acid equivalents (GAE) in the unit of mg of GAE/L.

Statistical analysis

Statistical analyses were conducted using Tukey's test in SPSS (17.0) (SPSS Statistics 17.0, Armonk, NY, USA) and differences were considered significant if $p < 0.05$.

Results and discussion

Antifungal activity of zein films containing *Thymus vulgaris, Laurus nobilis, Citrus sinensis* EOs and potassium sorbate

Zein films obtained from solutions containing various amounts (3–6% v/w) of *Thymus vulgaris* EO were prepared. The areas of clear zone around the discs calculated are shown in Table 1. Discs obtained from zein films without EO didn't have

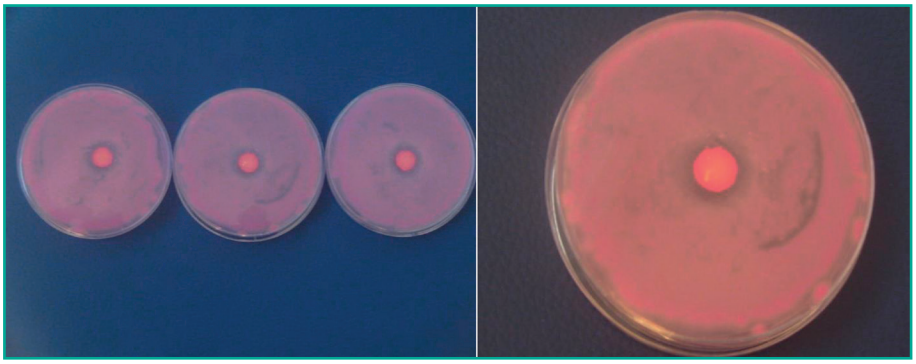

FIGURE 2: *Antifungal effect of films obtained from solutions containing 1.6% thyme and 1.6% laurel essential oil together on Aspergillus fumigatus.*

TABLE 1: *Inhibition zone that were formed by zein films containing thyme EO over mold and yeast*.*

Concentration of thyme EO in zein film solution (%v/w)	Amount of average EO in discs (mL*1000)	*Aspergillus fumigatus* Zone (cm)	*Paecilomyces variotii* Zone (cm)	*Aspergillus parasiticus* NRRL 465 Zone (cm)	*Candida kefyr* Zone (cm)	*Candida zeylanoides 1* Zone (cm)	*Candida zeylanoides 2* Zone (cm)	*Candida lambica* Zone (cm)	*Candida sake* Zone (cm)
3	1.8	All inhibited[1]	0.95±0.42	1.26±0.26	No zone formation	No zone formation	No zone formation	0.77±0.24^{b}	No zone formation
3.5	2.1	All inhibited	All inhibited	All inhibited	1.40±0.11^{b}	2.27±0.15^{a}	1.69±0.47^{bc}	All inhibited	2.08±0.08^{ab}
4	2.0	All inhibited	All inhibited	All inhibited	0.44±0.13^{c}	0.50±0.05^{c}	1.20±0.36^{c}	3.52±0.81^{a}	1.64±0.45^{b}
5	2.7	All inhibited	All inhibited	All inhibited	2.38±0.03^{a}	1.26±0.39^{b}	2.74±0.11^{a}	All inhibited	All inhibited
6	2.3	All inhibited	All inhibited	All inhibited	2.22±0.30^{a}	1.18±0.26^{b}	2.51±0.52^{ab}	All inhibited	2.36±0.1^{a}

* Results are expressed as mean ± standard deviation (n=3). [1] No growth was determined at medium. $^{a-c}$: Values in the same column with different superscripts indicate a statistically significant difference (P<0.05).

any inhibitory effect against molds and yeasts tested in this study.

Zein films obtained from solutions fortified with 3% thyme oil inhibited *Aspergillus fumigatus* but could not inhibit the *Aspergillus parasiticus* NRRL 465 and *Paecilomyces variotii* completely. However, zein films obtained from solutions with all other concentrations (3.5%, 4%, 5%, 6% v/w) inhibited all three; *Aspergillus parasiticus* NRRL 465, *Aspergillus fumigatus* and *Paecilomyces variotii* entirely. Also, when zein films obtained from solutions containing 3% thyme oil were applied to the yeasts, except for *C. lambica,* no inhibition zones were observed. The films obtained from solutions with 3.5% concentration of thyme oil showed clear zones around the all disks applied to yeasts. As the concentration of thyme oil in film solutions increased to 4%, 5%, 6% (v/w), completely inhibition of molds has been enhanced. As the concentration of thyme EO increased, the film solution became more fluid and also the flexibility and thickness of dried films increased. Therefore, the dissimilarity in the weight of discs affected the discs thickness and thereby the EO amount in the discs and release of EO from film. This can explain the slight deviations of the zone areas.

As can be seen from the Table 1, the most sensitive mold and yeast against the films containing thyme EO was found to be *A. fumigatus* and *C. lambica,* respectively.

Furthermore, *Laurus nobilis* EO was added to zein film solutions with 5 different concentrations (3%, 4%, 5%, 7% and 8% (v/w). As the concentration of this EO increased, the turbidity of film solution increased and the structure became more viscous when compared to film solutions including thyme EO. Also at the end of the drying period, increase in the fragility of dried films was observed. But films didn't show any antifungal activity at the stated concentration. *Citrus sinensis* EO in zein film solutions at the concentrations of 3%, 4%, 5%, 6%, 7% and 8% (v/w) also didn't have any antifungal activity. Film solutions became clear and it was more fluid when compared to film solutions including thyme EO. The fragility of films increased slightly by increasing the orange EO ratio. However films didn't show any antifungal activity at the stated concentrations.

Main reason of food spoilage is the microbial growth observed on food surfaces. It is important to provide controlled release of active substances to the food to inhibit the microbial growth for a long time (de Dicastillo et al., 2016). This is one of the important reasons why EOs were applied within films. Del Nobile et al. (2008) tested antimicrobial activity of thymol containing zein films and obtained good results. In different studies, antimicrobial activities of *Thymus vulgaris* and other EOs against mold, yeast and bacteria were reported (Behbahani et al., 2013; Nguefack et al., 2009). Our results show that zein films containing *Thymus vulgaris* EO are quiet effective against the tested molds and yeasts.

Antifungal activity of zein film containing combinations of EOs

Since zein film with thyme EO was determined as the most effective film against tested molds and yeasts; thyme EO was combined with laurel and/or orange EOs in zein film and their antifungal activities were investigated. Results were given in Table 2. Zein film obtained from solutions

TABLE 2: *Inhibition zone that were formed by zein films containing different combinations of EOs over molds and yeasts*.*

Concentration of EOs in zein film solution (%v/w)			Amount of average EO in discs (mL*1000)	*Aspergillus fumigatus* Zone (cm)	*Paecilomyces variotii* Zone (cm)	*Aspergillus parasiticus* NRRL 465 Zone (cm)	*Candida kefyr* Zone (cm)	*Candida zeylanoides 1* Zone (cm)	*Candida zeylanoides 2* Zone (cm)	*Candida lambica* Zone (cm)	*Candida sake* Zone (cm)
Thyme	Laurel	Orange									
3	3	–	2.5	All inhibited[1]	1.00^{d}-pfz-pfz	All inhibited	0.20-pfz-nz	Decrease of growth around disc	Decrease of growth around disc	3.43±2.04^{a}	0.41±0.21^{b}-nz
3	4	–	3.4	All inhibited	3.47±0.02^{b}	All inhibited	pfz-nz-nz	pfz-nz-nz	Decrease of growth around disc	2.85±0.09^{a}	0.30±0.04^{b}-nz
3	–	3	3.6	All inhibited	2.56±0.02^{c}	All inhibited	No zone formation	0.19±0^{b}-nz	pfz-nz-nz	3.31±0.39^{a}	0.52±0.12^{b}
3	1.5	1.5	3.9	All inhibited	5.78±0.47^{a}	1.54±0.61	No zone formation	0.49±0.07^{a}	0.07±0.01	2.91±0.37^{a}	2.54±0.29^{a}-ai

* Results are expressed as mean ± standard deviation (n=3). [1] No growth was determined at medium. ai: Full inhibition was determined at one of the parallel. pfz: Partially formed zone was determined at one of the parallel. nz: No zone was determined at one of the parallel. a–d: Values in the same column with different superscripts indicate a statistically significant difference (P<0.05).

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with concentration of 3% thyme EO completely inhibited only *A. fumigatus* however zein film obtained from solutions with concentration of 3% thyme and 3% laurel EO or zein film obtained from solutions with concentration of 3% thyme and 3% citrus EO together inhibited *A. parasiticus* NRLL 465 entirely as well as *A. fumigatus*. Also inhibition zone area over *P. variotii* increased with the combinational uses of EOs.

While zein film obtained from solutions containing 3% thyme EO showed antifungal activity only against *C. lambica*, zein film obtained from solutions with concentration of 3% thyme oil and 3% laurel EO together or zein film obtained from solutions with concentration of 3% thyme oil and 3% citrus EO together started to become effective against all other yeasts.

Activity of zein film prepared with combination of three EOs became more effective against *A. parasiticus* NRLL 465 than zein film including only 3% thyme EO, however could not inhibit completely. The antifungal activity of this film against *P. variotii* increased too much. And also it showed antifungal activity against all other yeasts except *C. kefyr*.

EOs from *Thymus vulgaris* and some other plants were evaluated in terms of their single and combined antibacterial or antifungal activities by Al-Bayati (2008) and Nguefack et al. (2012). In these studies synergistic effects of EOs were observed.

In our study, we obtained that combination of EOs used in zein films increased the antifungal activity. Especially binary uses of EOs can be used in the essays against molds and yeasts. It seems that the synergic effect of EOs increases the antifungal effect and also causes changes in the film structure that increases the amount of EOs released from film. By using the synergistic effects, we can both increase the antifungal activity of EOs and decrease the concentrations of EOs necessary to obtain required antimicrobial effect without changing the taste of foods.

Minimally inhibitory concentration of zein film against *Aspergillus fumigatus*

After the determination of the most effective EO in zein film against *Aspergillus fumigatus* as thyme oil and thyme-laurel oil mixture, minimally inhibitory concentration of zein film prepared with these EOs and potassium sorbate was calculated by using disc diffusion method. As a result, minimally concentration providing the inhibition of *Aspergillus fumigatus* was determined for the zein film obtained from solutions including only thyme EO as 1.7%, including thyme-laurel EO together as 1.6% and finally including potassium sorbate as 3.5% (Table 3, Table 4, Table 5).

While inhibition zone was observed in the films obtained from solutions containing 1.6% thyme EO and 1.6% laurel EO together, the inhibition zone was not determined with zein films obtained from solutions containing only 1.6% thyme EO. Laurel EO didn't show any antifungal effect when incorporated into zein film alone. However, when it was added with thyme EO, synergic effect of EOs has been observed. So for inhibition of *A. fumigatus*, concentration of 1.7% thyme EO in zein film was necessary. When 1.6% concentration of laurel EO was added with thyme EO, 1.6% thyme EO in zein film solution was enough for the inhibition of *A. fumigatus*. The minimally concentration of zein film containing potassium sorbate was determined as 3.5% (w/w).

Radical scavenging activity of EOs

In this study, besides the antimicrobial activities of *Thymus vulgaris, Laurus nobilis, Citrus sinensis* EOs in zein film, scavenging ability of these EOs was also tested.

IC_{50} values were found as 3.90, 54.27, 709.61, 0.004 g/L for *Thymus vulgaris, Laurus nobilis, Citrus sinensis* and gallic acid, respectively. These findings showed us that thyme EO had the highest radical scavenging activity among the tested EOs. The *Citrus sinensis* EO showed the lowest radical scavenging activity (Table 6).

Sokmen et al. (2004) determined IC_{50} value of *Thymus spathulifolius* EO as 243.2±7.20 µg/mL (0.2432 g/L). In the other study, IC_{50} value was found as 7.38 g/L for the EO from *Thymus vulgaris* L (Kodal Coskun et al., 2014). Since the genus *Thymus* comprises 215 species (Ložienė et al., 2007) the diversity in the antioxidant properties of genus is normal.

Limited number of studies on EOs from *Laurus nobilis* and *Citrus sinensis* were found. The results about IC_{50} values obtained in these studies (Singh et al., 2010; Yvon et al., 2012) were not compatible with our results. This can be explained by the plant diversity used by the researchers. EOs obtained from the same type of plant can have different antimicrobial activities because the geographic area, the climate and the genotype of the plant can vary. Also harvesting season, drying method and the distilled part of the plant play an important role in this diversity (Oussalah et al., 2007). This view can be also valid for the antioxidant activity of oils from the same plant.

Since free radicals play an important role in lipid peroxidation, the effect of EOs over free radicals has been determined and laurel EO was found to be suitable to use with thyme EO when binary uses were preferred.

TABLE 3: *Inhibition zone that were formed by zein films containing thyme EO over A. fumigatus.*

Concentration of thyme EO in zein film solution (%v/w)	Amount of average EOs in discs (mL*1000)	*Aspergillus fumigatus* Zone (cm)*
1.00	0.5	No zone formation
1.50	0.8	No zone formation
1.60	1	No zone formation
1.70	0.9	0.65±0.26^{c}
1.85	0.9	0.66±0.14^{c}
2.00	1.2	5.23±0.60^{b}
2.50	1.6	7.18±1.02^{a}

* Results are expressed as mean ± standard deviation. $^{a–c}$: Values in the same column with different superscripts indicate a statistically significant difference (P<0.05).

TABLE 4: *Inhibition zone that were formed by zein films containing thyme and laurel EO over A. fumigatus.*

Concentration of EOs in zein film solution (%v/w)		Amount of average EOs in discs (mL*1000)	*Aspergillus fumigatus* Zone (cm)*
Thyme	Laurel		
1.50	1.50	1.7	No zone formation
1.60	1.60	1.9	0.94±0.06^{a}
1.65	1.65	1.8	0.97±0.00^{a}

* Results are expressed as mean ± standard deviation. ^{a}: Values in the same column with different superscripts indicate a statistically significant difference (P<0.05).

TABLE 5: *Inhibition zone that were formed by zein films containing potassium sorbate over A. fumigatus.*

Concentration of potassium sorbate in zein film solution (%v/w)	Amount of average potassium sorbate in discs (g*1000)	*Aspergillus fumigatus* Zone (cm)*
1.60	0.9	No zone formation
2.50	1.8	No zone formation
3.50	2.3	0.65±0.26

* Results are expressed as mean ± standard deviation.

TABLE 6: *Radical scavenging activities (IC50 values) and total phenolic compound content of EOs.*

EOs and Gallic	IC_{50}* (g/L)	Total phenolic compounds** (mg of GAE/L)
Thyme	3.90[c]	273833±8209[a]
Laurel	54.27[b]	3157±33[b]
Orange	709.61[a]	3308±78[b]
Gallic acid	0.004[d]	

* Results are expressed as mean ± standard deviation (n=3). ** Results are expressed as mean ± standard deviation (n=4). [a–d]: Values in the same column with different superscripts indicate a statistically significant difference (P<0.05).

The total phenolic compound content of EOs

The amount of phenolic compounds were calculated as depending on gallic acid equivalents and found as 273833±20 mg/L for the EO of thyme, 3308±18 mg/L for the EO of orange and 3157±81 mg/L for the laurel EO. Although IC_{50} value of orange EO was found to be 13 times more than laurel EO, the amount of phenolic compounds of orange EO were higher (Table 6).

In the study of Sun et al. (2002), it was found that, there was a direct relation between the phenolic contents and total antioxidant activities in the tested 11 fruits. However, results found in some other studies displayed that there was slight (Anagnostopoulou et al., 2006) or no relation between total phenolic content and radical scavenging activity (Mohammadian et al., 2011). Thus, Wojdyło et al. (2007) reported that phenolic acids and flavonoids were typical major phenolics that are responsible for the antioxidant activity. The difference in the proportion of phenolic compounds may cause differences in the antioxidant activity. That's why even the total phenolic content is high, the total antioxidant activity may be low (Mohammadian et al., 2011; Li et al., 2006).

Conclusion

Zein films containing various amounts of *Thymus vulgaris* EO showed antifungal activity on different molds and yeasts. However zein films fortified with *Laurus nobilis* EO or *Citrus sinensis* EO didn't show any antifungal activity at applied concentrations. In our study, the antifungal activity of thyme EO was increased when used together with laurel EO or orange EO in zein films. This research showed that, the combinational uses of EOs in zein films increased their antimicrobial properties moreover the application of these zein films can replace the synthetic antioxidants and antimicrobial additives used in food industries. Furthermore, the flavor of the EOs chosen as antifungal additive should be compatible with the applied foods and should not generate any strange flavor.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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