



Antioxidant and antimicrobial properties of essential oils encapsulated in zein nanoparticles prepared by liquid–liquid dispersion method

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ABSTRACT

Two essential oils (EOs), thymol and carvacrol, with similar chemical structures were encapsulated in the nanoparticles of zein using the liquid–liquid dispersion method. Three different pH treatments were applied to study the nanoparticles' morphology, structure, antioxidant property, and antimicrobial activity. The nanoparticles for all treatments were well dispersed in water. After lyophilizing, samples from the acidic condition tended to form film, but the samples from the neutral and basic conditions formed nanoparticles. The antioxidant properties were analyzed by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) and Ferric ion spectrophotometric assay. DPPH• was reduced in the range of 24.8–66.8% depending on the formulation and more than 65% hydroxyl free radicals were quenched by samples. An *Escherichia coli* reduction of 0.8–1.8 log CFU/ml was achieved in the presence of nanoparticles encapsulating EOs.

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1. Introduction

Several recent foodborne illness outbreaks attributed to consumption of contaminated cantaloupes, eggs, raw meat and spinach (Anderson, Jaykus, Beaulieu, & Dennis, 2011; Gomes, Moreira, & Castell-Perez, 2011; Sheth et al., 2011) have awakened growing concern among customers, producers and government regarding food safety issues. Consequently, the need for more efficient antimicrobial agents is increasingly being recognized. The recognition of the potential toxicity of synthetic antimicrobials and the multiple health benefits attributed to many natural compounds has encouraged the quest for natural antimicrobial agents. Many essential oils (EOs), which are hydrophobic and volatile compounds derived from plants, have shown natural antifungal, insecticidal, antimicrobial, and antioxidant properties. Due to these characteristics, EOs have gained much attention and been studied for a long time (Kalemba & Kunicka, 2003; Seydim & Sarikus, 2006; Sivropoulou et al., 1996). Among EOs, thymol (5-methyl-2-isopropylphenol) and carvacrol (5-isopropyl-2-methylphenol) are predominant in oregano and thyme oil, respectively. Thymol and carvacrol both contain a phenolhydroxyl group, which makes them promising antimicrobials, because it allows them to disrupt the bacterial membrane leading to cell death (Di Pasqua, Hoskins,

Betts, & Mauriello, 2006). Both thymol and carvacrol are listed by the Food and Drug Administration (FDA) as food additives on the GRAS (Generally recognized as Safe) list, but they have poor solubility in water, which has limited their application as food additives. In this study, zein was used to form nanoparticles encapsulating EOs, which can be well dispersed in water while maintaining the EOs' antimicrobial and antioxidant properties.

Zein, a prolamine protein found in maize, has long been applied in food and pharmaceutical industries because of its film forming ability and superior biodegradability and biocompatibility. After cast drying, zein tends to form film under acidic treatment and particles under neutral and base treatments (Woods & Selling, 2007). Zein is soluble in aqueous ethanol solution with the ethanol content of 60–85 ml/100 ml. The liquid–liquid dispersion method makes use of the differential solubility of zein in ethanol and water to precipitate the zein nanoparticles, which encapsulate EOs that are codissolved in the aqueous ethanol along with zein (Zhong, Tian, & Zivanovic, 2009). When the stock solution of zein and EOs is sheared into bulk water, the solution can be emulsified into small droplets. The interdiffusion of alcohol and water results in a reduction of the ethanol concentration, which reduces the solubility of zein in the solution leading to its precipitation to form nano-scale particles with EOs encapsulated. Using this method, EOs can be dispersed in water and maybe applied in aqueous solution, such as fruit and vegetable juice beverages for antimicrobial and antioxidant benefits.

Zein was GRAS and classified as a food-grade ingredient by the FDA and it has been used in many applications, including but not

Abbreviations: T4, T6.5; T10, C4; C6.5, C10.

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limited to drug delivery (Chen & Subirade, 2009; Lai & Guo, 2011), nutrition protection in food (Onal & Langdon, 2005; Saremnezhad, Azizi, Abbasi, & Ahmadi, 2011), antioxidant (Gucbilmez, Yemenicioglu, & Arslanoglu, 2007; Kong & Xiong, 2006; Matsumura, Andonova, Hayashi, Murakami, & Mori, 1994) and emulsifier (Cabra, Arreguin, Vazquez-Duhalt, & Farres, 2007). Its properties of biodegradability (Wang, Gong, & Wang, 2008), in vivo biocompatibility (Yao, Li, & Song, 2009) and different morphology/solubility under different pH conditions (Cabra, Arreguin, Vazquez-Duhalt, & Farres, 2006) add to its versatility and exceptional suitability for a variety of uses. As a protein, zein has both hydrophobic and hydrophilic zones, which can form diverse bonds to encapsulate Gitoxin for a sustained-release form (Muthuselvi & Dhathathreyan, 2006). The unique solubility of zein in ethanol aqueous solution was used to encapsulate EOs like those of oregano, red thyme and cassia (Parris, Cooke, & Hicks, 2005; Zhong & Jin, 2009). EOs encapsulated in zein nanoparticles have been studied recently for their antioxidant activity and release kinetics (Del Nobile, Conte, Incoronato, & Panza, 2008). However, studies to improve the solubility of EOs through nanotechnology have not been found. Therefore, this study was carried out using zein nanoparticles to encapsulate EOs for the purpose of improving their solubility while retaining their antimicrobial and antioxidant properties. The EOs used in this research are thymol and carvacrol, which have the same chemical composition but different position of hydroxyl groups on the phenolic ring. The effect of different pH conditions on solubility and other properties was also investigated.

2. Materials and methods

Zein sample with a minimum protein content of 97 g/100 g was obtained from Showa Sangyo (Tokyo, Japan). All chemicals used, including ethanol, ethyl acetate, hydrochloride acid, sodium hydroxide, hydrogen peroxide, phosphate buffered saline (PBS), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) and 1,10-phenanthroline-iron (II) were purchased from Sigma–Aldrich (St. Louis, MO, USA). *Escherichia coli* was purchased from ATCC (# 53323 Manassas, VA, USA), and lysogeny broth (LB) medium was bought from Difco (Franklin Lakes, NJ, USA).

2.1. Sample preparation

Zein and either of the EOs (thymol/carvacrol) were dissolved in 70 ml/100 ml aqueous ethanol solutions separately to obtain final concentrations of 50 mg/ml and 20 mg/ml, respectively. The stock solutions of zein and EOs were sheared into bulk deionized water using a homogenizer at 17,500 rpm for 2 min. Nitrogen was pumped into solution for 1 h to remove the ethanol from the system using a nitrogen evaporator (N-EVAP™, Organomation Associates, Inc, MA, USA) and then the dispersion was lyophilized (RVT 4104-115, Thermo Savant, Waltham, MA, USA) to yield dry powder. Samples treated under different pH conditions were prepared, including acidic pH (4) adjusted with hydrochloride acid, neutral pH (6.5), and basic pH (10) adjusted with sodium hydroxide.

2.2. Encapsulation efficiency (EE)

Ethyl acetate was used to extract thymol/carvacrol from dry samples of zein nanoparticles encapsulating thymol/carvacrol because thymol/carvacrol is soluble in ethyl acetate while zein is not. The standard curve was obtained by dissolving pure thymol/carvacrol in ethyl acetate at a series of mass to volume ratios and reading the absorbance of these solutions at 263 nm (for thymol) or 275 nm (for carvacrol) using the UV–visible spectrophotometer (Beckman Coulter, DU-730, Fullerton, CA, USA). Either pure

thymol/carvacrol was mixed and diluted with ethyl acetate to obtain standard calibration curve.

The 5 mg dry samples of zein nanoparticles encapsulating thymol or carvacrol were dissolved in 10 ml ethyl acetate in an ultrasonic bath for 1 h and then filtered with a 1 µm syringe filter before spectrophotometric assay. The encapsulation efficiency (EE) of essential oils was determined by the following equation (Xiao, Gommel, Davidson, & Zhong, 2011).

$$EE(\text{g}/100 \text{ g}) = \frac{\text{Mass of EOs in nanoparticles}}{\text{Mass of EOs added}} \times 100 \quad (1)$$

2.3. Particle size and morphology

Samples (including the fresh prepared ones, ones with removal of ethanol, and the re-dispersed ones after lyophilizing) were measured by a dynamic light scattering instrument (DLS, BI-200SM, Brookhaven Instruments Corporation, Holtsville, NY, USA).

The morphology of samples with different EOs encapsulated under different pH conditions were compared. All measurements were taken at 20 °C and refractive indices for various aqueous ethanol solutions were applied for autocorrelation to calculate the effective diameter.

Scanning electron microscopy (SEM, SU-70 SEM, Hitachi, Pleasanton, CA, USA) was used to observe the morphology of samples under different pH conditions. After lyophilizing, dry samples were adhered to conductive carbon tapes (Electron Microscopy Sciences, Ft. Washington, PA, USA), and mounted onto specimen stubs coated with a thin (<20 nm) conductive gold and platinum layer using a sputter-coater (Hummer XP, Anatech, CA, USA). Digital images of the samples were obtained and representative images are presented.

2.4. Fourier Transform Infrared Spectroscopy (FTIR)

FTIR was used to document the changes in chemical structure of zein, EOs and nanoparticle samples. The spectra was acquired at 400–4000 cm^{−1} wavenumbers with 1 cm^{−1} resolution utilizing a Jasco 4200 series FTIR spectrophotometer (Jasco Inc., Easton, MD, USA) equipped with a diamond ATR cell.

2.5. Solubility test

Solubility of EOs (thymol/carvacrol) was measured as follows (Kim, Jeong, Choi, Roh, Kang, Jang, et al. 2006; Trapani, Sitterberg, Bakowsky, & Kissel, 2009), 200 mg EOs was dissolved in 20 ml 70 ml/100 ml aqueous ethanol and then dispersed into 40 ml DI water using a homogenizer at 17,500 rpm at different pH values (4, 6.5 and 10). The ethanol was removed using a nitrogen evaporator (N-EVAP™, Organomation Associates, Inc, MA, USA) for 1 h. The insoluble EOs were removed from the aqueous solution by gravity filtration using filter paper (Whatman No. 1, VWR International, LLC, PA, USA) and then the soluble EOs were extracted with ethyl acetate. The ethyl acetate containing EOs was diluted 60 times before measuring absorbance with a UV–visible spectrophotometer (DU-730, Beckman Coulter, Fullerton, CA, USA). The standard curve obtained in part 2.2 was used to determine the maximum concentration of EOs in the water under different pH values. The solubility enhancement was obtained by comparing EO water solubility before and after encapsulation in nanoparticles.

2.6. Antioxidant activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) and 1,10-phenanthroline-iron (II) complex free radical-scavenging assays were used for the measurement of antioxidant activity of different samples.

For the DPPH• assay, 0.1 mmol/L DPPH• solution was prepared with 4 mg/ml (effective concentrations of EOs in samples) sample solution prepared in 70 ml/100 ml aqueous ethanol. After continuous incubation in the dark at 4 °C for varying lengths of time (0.5 h, 1 h, 12 h and 24 h), the DPPH radical scavenging activity of the sample was assessed by measuring absorbance at 517 nm against 70 ml/100 ml aqueous ethanol as a blank. The control was prepared by replacing the 4 mg/ml sample solution with same amount of 70 ml/100 ml aqueous ethanol. Also zein without EOs was included as a comparison.

For the 1,10-phenanthroline-iron (II) assay, samples were evaluated for their ability to prevent 1,10-phenanthroline-iron(II) complex $[\text{Fe}(\text{phen})_3]^{2+}$ from oxidation by hydrogen peroxide. Since oxidation of Fe (II) was pH sensitive, 10 mmol/L phosphate buffered saline (PBS) was also used. For each test, 1 ml of 1,10-phenanthroline solution in deionized water, 1 ml of FeSO_4 solution in deionized water and 1 ml of 0.025 ml/100 ml H_2O_2 were mixed with 1 ml of 4 mg/ml (effective concentrations of EOs in samples) sample solution in deionized water and 2 ml of PBS. In the negative control group, 1 ml of PBS was used to replace the sample solution. Also 1 ml PBS was used to replace the 1 ml H_2O_2 in the positive control group. The optical density of each group was measured using the spectrophotometer at 509 nm against a 10 mmol/L PBS blank.

The hydroxyl free radical clearance (percentage) is defined in the Equation below, where A is the Absorbance at 509 nm.

Hydroxyl Free Radical Clearance (%)

$$= \frac{(A_{P\text{-control}} - A_{\text{Sample}})}{(A_{P\text{-control}} - A_{N\text{-control}})} \times 100 \quad (2)$$

After the 12 h incubation, the hydroxyl free radical clearance (percentage) was obtained for each sample.

2.7. Antimicrobial activity

Ability of different treatments to inhibit growth of non-pathogenic *E. coli* (ATCC# 53323) was evaluated. The *E. coli* used in this experiment is non-pathogenic, it was selected because of its similarity to well known pathogenic bacteria responsible for many foodborne illness outbreaks and clinical infections. *E. coli* were inoculated on lysogeny broth (LB) and samples of the 6 treatments were dispersed in LB medium at an EO to broth ratio of 0.02 g/100 g. LB inoculated with same amount of *E. coli* without addition of antimicrobial agents was treated as the control. The optical density of each inoculated broth was measured at 550 nm using a spectrophotometer. The optical density result can be converted into log CFU/ml, after 12, 24, 36 and 48 h at 35 °C to obtain the inhibition to growth of *E. coli* in comparison to the control.

2.8. Statistical analysis

The data, reported as mean \pm standard error are from experiments conducted in triplicate. Experimental statistics were performed using a SAS software (Version 9.2, SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) was used to check the assumptions of variance homogeneity and normality and compare the treatment means. Antioxidant activity was analyzed according to a two factor model with 2 levels of EO (thymol/carvacrol) and 3 levels of pH (4, 6.5 and 10) using Tukey's multiple comparison test. The free radical scavenging test was analyzed according to a 3 factor model, with EO, pH and reaction time as the 3 factors using Tukey's Studentized Range (HSD) test to make selected pairwise means comparisons. Differences were considered to be statistically significant for *P*-values less than or equal to $\alpha = 0.05$.

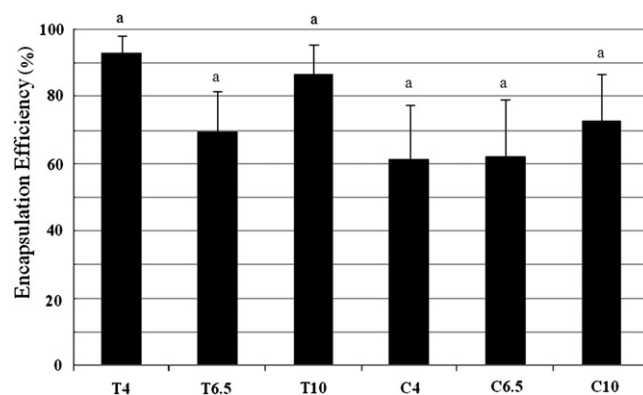


Fig. 1. Encapsulation efficiency by extracting EOs from samples with ethyl acetate. T4, T6.5 and T10 are samples encapsulating thymol under pH = 4, 6.5 and 10 respectively. C4, C6.5 and C10 are samples encapsulating carvacrol under pH = 4, 6.5 and 10 respectively. There is no significant difference between each other.

3. Results and discussion

3.1. Encapsulation efficiency (EE)

Fig. 1 shows results of the EE, more than 50% of EOs was encapsulated in zein for all treatments using this method. There is no significant difference between the EE for thymol and carvacrol. Carvacrol has slightly higher solubility than thymol in water, which may result in more release of carvacrol into water, but the results show that there is no difference between the solubilities of all the samples. Since thymol and carvacrol are both volatile essential oils, they were expected to be partially evaporated under low pressure during lyophilizing. Therefore, the real EE should be a little higher than the obtained experimental data. Similar studies about the effect of volatility on encapsulation have shown that more than 90% of non-volatile fish oil could be encapsulated by zein, while only 65–75% of volatile essential oils could be encapsulated (Zhong et al., 2009).

3.2. Particle size

DLS was used to determine the particle size before lyophilizing, and after lyophilizing and re-dispersion in water (Table 1). The particle sizes of all treatments were kept below 330 nm before lyophilizing and raised a minimum of 160 nm to the range between 430 nm and 740 nm after re-dispersion. The exception was the treatment C10, which had particle sizes of 52 nm and 205 nm before and after lyophilizing, respectively. The polydispersity index was relatively low (below 0.3), which ensured the reliability of data from DLS (Dobrynin, Leibler, 1997). Particle sizes of all treatments increased after lyophilizing and re-dispersion, indicating that some of the nanoparticles formed by dispersion aggregate during lyophilizing. The samples still remained less than 800 nm which ensured the good solubility and application in water solution.

Table 1
Effective diameter and polydispersity of different treatments.

EOs	pH	Effective diameter (nm)		Polydispersity	
		Before lyophilizing	Re-dispersion	Before lyophilizing	Re-dispersion
Thymol	4	328.1 \pm 21.5	732.2 \pm 25.3	0.259 \pm 0.023	0.145 \pm 0.011
	6.5	269.4 \pm 32.9	432.1 \pm 43.3	0.136 \pm 0.029	0.234 \pm 0.021
	10	259.4 \pm 59.6	654.2 \pm 13.2	0.199 \pm 0.032	0.298 \pm 0.034
Carvacrol	4	293.6 \pm 25.4	490.2 \pm 18.2	0.184 \pm 0.025	0.321 \pm 0.033
	6.5	223.2 \pm 42.3	543.8 \pm 34.2	0.270 \pm 0.008	0.117 \pm 0.020
	10	51.9 \pm 15.2	205.0 \pm 43.3	0.183 \pm 0.012	0.229 \pm 0.032

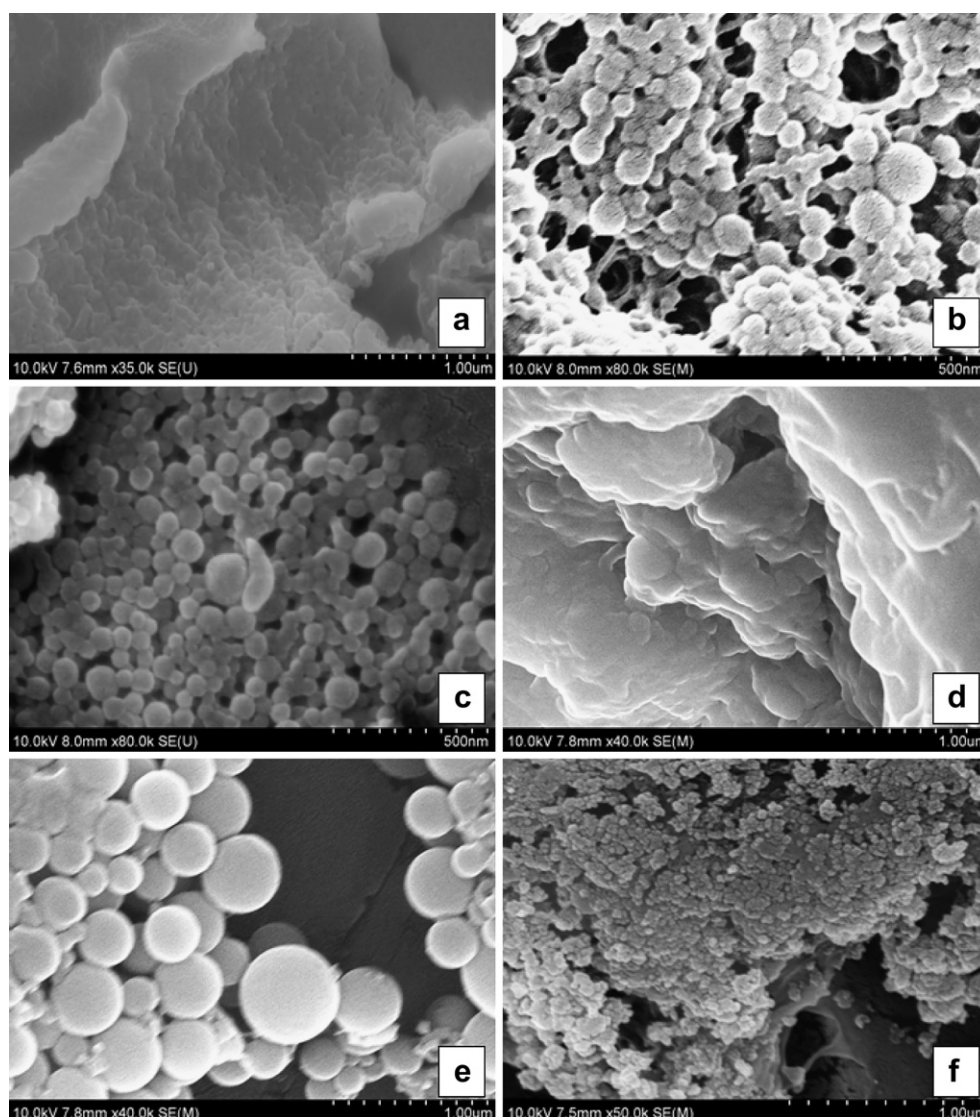


Fig. 2. SEM images for samples encapsulating EOs under different pH conditions after lyophilizing, (a), (b) and (c) are encapsulating thymol under pH = 4, 6.5 and 10 respectively. (d), (e) and (f) are encapsulating carvacrol under pH = 4, 6.5 and 10 respectively.

3.3. SEM image

SEM images can directly provide information on the particle size and morphology of samples. Fig. 2 shows the SEM images of zein encapsulating EOs under different pH conditions. It can be seen that the sample morphology was dependent only on the pH no matter which EOs are encapsulated. The lyophilized samples from acidic solutions formed films (T4 and C4),¹ while nano-scale particles were formed after lyophilizing from neutral and basic solutions with the diameters ranging from 100 nm to 500 nm (T6.5, C6.5, T10 and C10). Zhang, Luo, and Wang (2011) also found that after lyophilizing, zein from acidic solution formed continuous phase of film and zein from near neutral and basic solutions formed dispersed particles.

The phase separation (liquid–liquid dispersion) method used in this study was quicker, did not require temperature control, and

reagents used to produce nanoparticles were more economical than for other preparation methods, such as chemical conjugation method (Suzuki et al., 1989) and solid-in-oil-in-water (S/O/W) emulsion process (Morita, Sakamura, Horikiri, Suzuki, & Yoshino, 2000). Fish oil has been encapsulated by this method with little change in particle size (350–450 nm) before lyophilizing and after re-dispersing in acetic acid/acetate buffer (Zhong et al. 2009). EOs (thymol and cinnamaldehyde) have also been encapsulated by phase separation with 0.01 ml/100 ml silicone fluid, which resulted in samples between 100 and 250 nm after lyophilizing (Parris et al. 2005).

3.4. FTIR spectrum

FTIR provides information of secondary structures of proteins (Bonnier et al., 2008) and also is used for EOs analysis (Vera & Chane-Ming, 1999). Only the treatments under neutral pH are presented because different pH conditions had no effect on FTIR results.

As shown in Fig. 3, the peak of zein hydroxyl group (3287.07 cm^{-1}) merges with that of thymol and carvacrol phenolhydroxyl groups,

¹ Samples encapsulating thymol under different pH conditions (pH 4, 6.5 and 10) were named T4, T6.5 and T10. Similarly, C4, C6.5 and C10 were used for samples encapsulating carvacrol under different pH conditions (pH 4, 6.5 and 10.5).

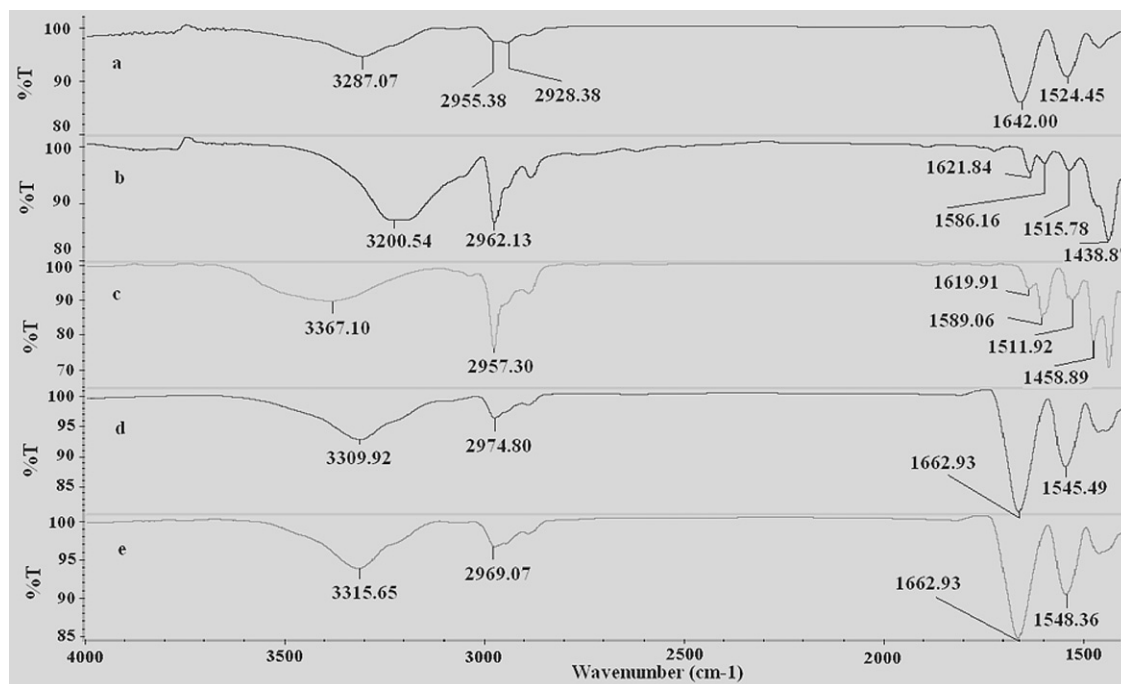


Fig. 3. FTIR spectrum: (a) zein (b) thymol (c) carvacrol (d) zein nanoparticles encapsulating thymol under pH = 6.5 (e) zein nanoparticles encapsulating with carvacrol under pH = 6.5.

specifically 3296.71 cm^{-1} for T6.5 and 3300.57 cm^{-1} for C6.5. The sharp peaks at 2955.38 cm^{-1} and 2928.38 cm^{-1} representing C–H stretching from CH_3 and CH_2 functional groups (Zhang, Luo, & Wang, 2010) merged with peaks at 2962.13 cm^{-1} and 2957.3 cm^{-1} for thymol and carvacrol, respectively. In this case, the functional groups responsible for the C–H stretching are the methyl and isopropyl groups on the phenolic rings of EOs (thymol/carcacrol). The four peaks specific to the phenolic ring at wavenumbers ranging from $1622\text{--}1458\text{ cm}^{-1}$ for thymol/carcacrol disappeared with the effect of the similar positions of Amide I and Amide II of zein (1642 cm^{-1} and 1524.45 cm^{-1} , respectively) (Gillgren, Barker, Belton, Georget, & Stading, 2009; Kumar, Tripathi, Mistry, & Bajpai, 2009).

All information reflects that no new peaks appeared and that zein and EOs were mixed together physically without any chemical reaction. The structure and function of EOs were not changed in this process, suggesting retention of antioxidant and antimicrobial properties.

3.5. Solubility enhancement

The solubility of EOs (thymol/carcacrol) was enhanced by zein encapsulation. All encapsulation treatments regardless of pH conditions showed increased solubility of the EOs ranging from 4 to 15 fold (as shown in Fig. 4). Different pH conditions affected the degree of solubility enhancement. The data suggest that solubility of EOs was enhanced least at pH 10. This result can be explained by the deprotonation of the phenolichydroxyl group of EOs under high pH. Some studies also focused on improving the solubility of EOs. Complexation with cyclodextrin has been used to increase the solubility of EOs up to 10 fold (Samperio, Boyer et al. 2010). Water-soluble chitosan (WSC), a polymeric amphiphile favored formation of self-aggregates to carry thymol (Hu, Du, Wang, & Feng, 2009). However, EOs in our study were made water soluble neither by forming a complexation, nor by using an amphiphile, but rather by encapsulated in zein nanoparticles.

3.6. Antioxidant capacity

3.6.1. DPPH• assay

All samples showed antioxidant capacity against the 70 ml/100 ml aqueous ethanol as blank (Fig. 5). The longer the incubation period, the lower the absorbance of all the samples, as a result of having more time to quench free radicals and bring about a more complete color change. All the samples encapsulating thymol could scavenge free radicals more quickly than those with carvacrol, except C6.5. The mechanism of this assay was based on the reduction of DPPH• by the antioxidant into a colorless substance. After 24 h inoculation, 37.5–75% of DPPH• had been reduced.

The antioxidant activity of EOs including thymol and carvacrol has been studied quantitatively by other researchers. Amiri et al. (2011) reported that about 0.1 mg/ml of thymol and 0.3 mg/ml of carvacrol could provide 50% inhibition of DPPH•. In our study

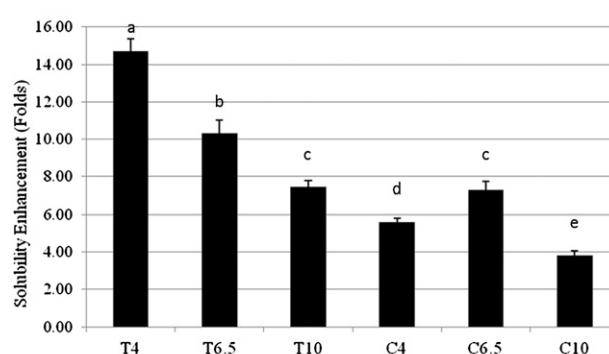


Fig. 4. Enhancement of solubility of EOs after encapsulation in each treatment. T4, T6.5 and T10 are samples encapsulating thymol under pH = 4, 6.5 and 10 respectively. C4, C6.5 and C10 are samples encapsulating carvacrol under pH = 4, 6.5 and 10 respectively. Means marked with different letters indicated a significant difference between each other upon the ANOVA Tukey's Studentized Range Test ($P < 0.05$).

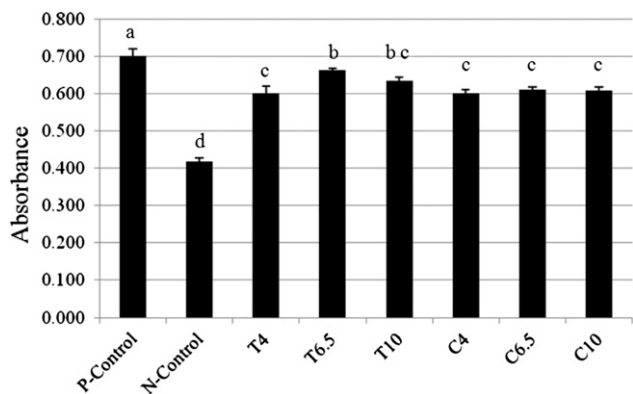


Fig. 5. DPPH• radical scavenging results of different samples over time by absorbance at 517 nm against 70 ml/100 ml aqueous ethanol as blank. T4, T6.5 and T10 are samples encapsulating thymol under pH = 4, 6.5 and 10 respectively. C4, C6.5 and C10 are samples encapsulating carvacrol under pH = 4, 6.5 and 10 respectively. Means marked with different letters indicated a significant difference between each other upon the ANOVA Tukey's Studentized Range Test ($P < 0.05$).

0.67 mg/ml zein encapsulated carvacrol at pH 4 and 10 inhibited 50% of DPPH• and carvacrol at pH 6.5 and thymol at all pH achieved more than 50% inhibition of DPPH•. Zein alone was able to inhibit the free radicals in a related study by Zhang et al. (2011). The results indicated that zein had slight antioxidant capacity (less than 25% DPPH• reduced after 24 h). The zein was also measured in this study at the same concentration. After deducting zein's contribution to the antioxidant capacity, DPPH• was reduced by an additional 24.8–66.8% among different treatments.

3.6.2. Ferric ion spectrophotometric assay

The antioxidant capacity of samples was further demonstrated, by the ferric ion spectrophotometric assay. Results from Fig. 6 showed that the $[\text{Fe}(\text{o-phen})_3]^{2+}$ complex was protected by samples from being oxidized by hydrogen peroxide. All samples showed greater antioxidant capacity than the PBS control. PBS was used to prevent ferric or ferrous precipitates, because the ferric ion assay is pH sensitive (Fig. 7).

The Hydroxyl Free Radicals Clearance Percentage (HFRC) data were obtained from Equation (2), and listed in Table 2. There was little difference in HFRC measurement among EOs encapsulated under different pH conditions. In all treatment groups, hydroxyl free radicals were cleared by 60–90%.

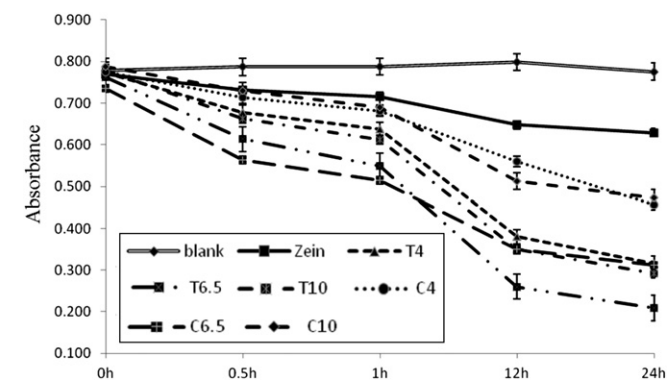


Fig. 6. Antioxidant activity of different samples against hydrogen peroxide and the formation of hydroxyl radicals by absorbance at 509 nm against 10 mM PBS as blank. T4, T6.5 and T10 are samples encapsulating thymol under pH = 4, 6.5 and 10 respectively. C4, C6.5 and C10 are samples encapsulating carvacrol under pH = 4, 6.5 and 10 respectively.

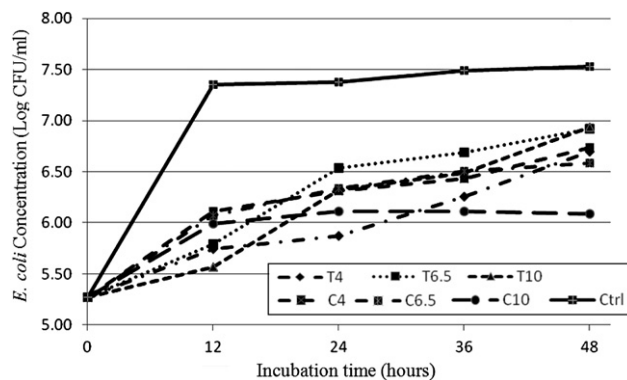


Fig. 7. Growth curve of *E. coli* in LB medium with different samples under 35 °C. T4, T6.5 and T10 are samples encapsulating thymol under pH = 4, 6.5 and 10 respectively. C4, C6.5 and C10 are samples encapsulating carvacrol under pH = 4, 6.5 and 10 respectively.

The antioxidant activity analysis required at least two methods, because of the complexity of the phytochemical reactions (Schlesier, Harwat, Bohm, & Bitsch, 2002). Both DPPH• and Ferric ion assays showed that encapsulation did not have much effect on the EOs' antioxidant activity. Thymol antioxidant activity was consistently superior to that of carvacrol, possibly due to greater steric hindrance of the thymol phenolic group (Viuda-Martos et al., 2010; Yanishlieva, Marinova, Gordon, & Raneva, 1999).

3.7. Antimicrobial activity

The in vitro antimicrobial activity of thymol/carvacrol against *E. coli*, a Gram-negative bacterium, was evaluated by determining the growth curve in LB medium. Both thymol and carvacrol exhibited interesting antimicrobial activity against *E. coli* after the 48 h's incubation in LB medium at 35 °C (Du et al., 2008). Thymol and carvacrol both significantly decreased the concentration of *E. coli* by 0.8–1.8 log CFU/ml compared to the control. Although some studies suggest that the release of EOs from zein films can be affected by loaded spelt bran and film's thickness (Mastromatteo, Barbuzzi, Conte, & Del Nobile, 2009), zein nanoparticles in this study were found to quickly release all the encapsulated EOs into LB medium. This rapid release is probably the result of the digestion of the zein nanoparticles by *E. coli* (Imam & Gordon, 2002).

Thymol and carvacrol have a similar chemical structure, which is responsible for their antimicrobial activity; both contain delocalized electrons and hydroxyl groups on benzene rings. Lambert, Skandamis Coote, & Nychas (2001) proposed that these EOs can break the equilibrium of inorganic ions and pH homeostasis inside the cytoplasm of bacteria. Ultee, Bennik, & Moezelaar (2002) further demonstrated that carvacrol can act as a transmembrane cation carrier by diffusing through the cytoplasmic membrane, releasing its hydroxyl proton into the cytoplasm, and transporting a potassium ion (or other cations) back across the membrane into the external environment.

Table 2
Hydroxyl free radicals clearance (%).

EOs	pH	Hydroxyl free radicals clearance %
Thymol	4	64.7 ± 3.9 a
	6.5	86.6 ± 8.1 b
	10	76.7 ± 8.3 ab
Carvacrol	4	65.0 ± 0.3 b
	6.5	68.2 ± 6.8 b
	10	67.8 ± 6.9 b

Means marked with different letters indicate a significant difference between each other upon the ANOVA Tukey's test ($P < 0.05$).

Thymol and carvacrol showed different temporal patterns of antimicrobial ability, thymol was consistently more efficient in limiting the growth of *E. coli* than carvacrol under same pH conditions in the short term (less than 12 h), but in the long term, carvacrol achieved better inhibition than thymol except T4 and C4 (as indicated by data at 48 h inoculation). Although Ultee et al. (2002) reported that the position of hydroxyl group on the phenolic ring has no effect on the antimicrobial ability, our results showed a significant difference ($P < 0.05$). We hypothesize that the position of hydroxyl group may cause differences in polarity and steric hindrance between the thymol and carvacrol molecules, resulting in different ability to interact with *E. coli* cells.

4. Conclusions

Essential oils have been studied extensively and there is much literature regarding their desirable antioxidant and antimicrobial properties, however, poor water solubility limits their application. Encapsulation of EOs in zein nanoparticles allows their dispersion in water, which greatly enhances their potential for use in food preservation and control of human pathogenic bacteria. Our study has demonstrated that encapsulating EOs in zein nanoparticles can enhance their solubility up to 14 fold without hindering their ability to scavenge free radicals or to control *E. coli* growth. Results from this study support the use of nanoencapsulation to facilitate the application of EOs in food preservation.

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