ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF THYME AND CLOVE ESSENTIAL OILS AND APPLICATION IN MINCED BEEF

HATICE ZENGIN and AYSE HANDAN BAYSAL[1]

Department of Food Engineering, Izmir Institute of Technology, Urla, Izmir 35437, Turkey

[1]Corresponding author.
TEL: +90-232-7506187;
FAX: +90-232-7506196;
EMAIL: handanbaysal@iyte.edu.tr

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ABSTRACT

In this study, thyme and clove essential oils (EOs) were studied for their chemical composition, antioxidant, antiradical and antibacterial activity and application in ground beef. Carvacrol (75.27%) and eugenol (75.2%) were detected by gas chromatography-mass spectrometry analysis as the main components of thyme and clove EOs, respectively. Broth microdilution method showed all bacteria inhibited by the EOs, while *Shewanella putrefaciens* and *Listeria innocua* were the most resistant bacteria to thyme and clove EOs, respectively. EO treatment restricted the growth of artificially inoculated *Salmonella typhimurium* and native *Coliforms* in the ground beef. Antioxidant activity determined by ferric-reducing antioxidant power and 1,1 diphenyl-2-picrylhydrazyl methods demonstrated that clove EO had higher *in vitro* antioxidant activity than thyme EO. Similar results were obtained in ground beef application using 2-thiobarbituric acid value. EOs of clove (2MIC) exerted remarkable higher antioxidant activity in ground beef than EOs of thyme (4MIC), which represent valid alternative antioxidant in meat products.

PRACTICAL APPLICATIONS

Plant essential oils (EOs) serve as a "safe" alternative to chemical or synthetic antimicrobials and antioxidants to struggle with the foodborne pathogens or spoilage organisms, inhibiting lipid oxidation and thus extending shelf life. Antioxidant activity determined by ferric-reducing antioxidant power and 1,1 diphenyl-2-picrylhydrazyl methods showed that clove EO had higher *in vitro* antioxidant activity than thyme EO. Results obtained using 2-thiobarbituric acid value in ground beef application were found similar. EOs of clove (2MIC) exerted higher antioxidant activity in ground beef than EOs of thyme (4MIC). Therefore, EOs could be a valid alternative antioxidant in meat products.

INTRODUCTION

Natural products such as plant essential oils (EOs) have uses in human health such as functional food, food additives, medicine, nutritional supplements and cosmetic manufacturing. Antioxidants are effective for inhibiting different human diseases due to their antiradical, antioxidant and antimicrobial properties. Therefore, investigation of bioactive compounds particularly polyphenols from natural plant sources including herbs and spices has an increasing trend. Plant EOs have antimicrobial, antioxidant and antimutagenic activities, and potential beneficial effects on certain health conditions. These generally recognized as safe nature substances inhibited lipid oxidation in foods, thereby serving as natural additives in foods and food products (Burt 2004; FDA 2013).

Thyme (*Thymus* sp.) and clove (*Syzygium aromaticum*) EOs are of much attention due to their high content and wide spectrum of phenolic compounds, antimicrobial and antioxidant properties, and potential for use in meat and meat products (Gutierrez *et al.* 2008; Barbosa *et al.* 2009; Gutierrez *et al.* 2009; Jayasena and Jo 2013; Bensid *et al.* 2014). The use of EOs is reported in the literature to improve shelf life of meat (Lucera *et al.* 2012; Jayasena and Jo 2013).

To the best of our knowledge, there is no data on antioxidant, antiradical and antimicrobial activities of thyme and clove EOs *in vitro* and on their antimicrobial and antioxidant effects in a real food system such as minced beef. The purpose of this study was to identify the main phenolic compounds present in thyme and clove EOs and determine their antioxidant and antimicrobial effects by *in vitro* and *in vivo* approaches. Total antioxidant activity was determined by ferric-reducing antioxidant power (FRAP) and 1,1 diphenyl-2-picrylhydrazyl (DPPH) methods. The composition and total phenolic content (TPC) were analyzed by gas chromatography-mass spectrometry (GC-MS) analysis and Folin–Ciocalteu assay, respectively. The antibacterial activity was evaluated by inhibition of *in vitro* cell cultures of meat borne spoilage and pathogen bacteria, namely, *Staphylococcus aureus*, *Listeria innocua*, *Carnobacterium divergens*, *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Shewanella putrefaciens* and *Serratia liquefaciens*. Antimicrobial and antioxidant effects of the EOs in minced beef were determined by the evaluation of 2-thiobarbituric acid (TBA) and color parameters and by the efficacy to eliminate or control artificially inoculated *Sa. typhimurium* and its native microbial flora, respectively, during storage under refrigeration (4C) aerobically.

MATERIALS AND METHODS

EOs

Commercial food-grade thyme and clove EOs were purchased from native producer. All the chemicals used were of analytical grade and they were purchased from Sigma Chemical Co. (St. Louis, MO). Different concentrations of EOs were prepared in ethanol daily. The final concentration of ethanol was not more than 1% in broth. Because dimethyl sulphoxide (DMSO) is toxic and cannot be used in food systems, ethanol was chosen as solvent (Burt *et al.* 2007).

Microbial Strains

Pathogenic, nonpathogenic and spoilage-related bacteria were handled during the study. Spoilage-related bacteria were obtained from the United States Department of Agriculture Agricultural Research Service Culture Collection (NRRL). Others were obtained from the Department of Food Engineering, Izmir Institute of Technology. Gram-positive and -negative bacterial strains used in the study (their origins) were *St. aureus* RSSK 01009 (clinical), *L. innocua* NRRL B-33314 (turkey/ham, deli sticks), *C. divergens* NRRL B-14830 (minced beef), *E. coli* O157:H7 ATCC 700728 (unknown), *Sa. typhimurium* CCM5445 (unknown), *Sh. putrefaciens* NRRL B-951 (unknown) and *Se. liquefaciens* NRRL B-41553 (ground beef). *E. coli* O157:H7, *Sa. typhimurium*, *St. aureus* and *L. innocua* strains were grown in nutrient broth (NB). *Sh. putrefaciens* and *Se. liquefaciens* were first propagated in tryptic soy broth, whereas *C. divergens* was grown in YG broth. After first propagation all bacteria were transferred to NB and growth was observed. NB medium was used for all the experiments.

Bacterial Suspensions

Cultures were grown in appropriate media and incubation conditions. Then bacterial suspensions were adjusted equivalent to 0.5 McFarland standard by using a Densitometer (DEN-1, HVD Life Sciences, Vienna, Austria) (approximately 7–8 $\log_{10}$/mL) and one more 10-fold dilution was performed in broth medium.

Determination of Antimicrobial Activity by Broth Micro- and Macrodilution Methods

For broth microdilution, 20 μL of bacterial suspension was added to the wells of a sterile 96-well microtiter plate containing 180 μL of twofold diluted EOs. Control wells were prepared with culture medium inoculated with bacterial suspension and also EOs without inoculation. Plates were incubated for 24 h and turbidity was determined by a microplate reader (Varioskan Flash, Thermo) at 600 nm with 30-min interval (Klančnik *et al.* 2010). After the incubation period, 100 μL of samples was taken from each wells and spread on agar plates to check the growth of bacteria. The MICs of EOs were recorded as the lowest concentration where no viability was observed in the wells of 96-microwell plates after incubation for 24 h. For broth macrodilution (Klančnik *et al.* 2010), 100 μL of bacterial suspensions was inoculated to 900 μL of growth media already containing desired concentration of EOs and incubated by shaking for 24 h at appropriate incubation temperatures. After 24 h 100 μL of sample was directly spread on agar plates and growth of colonies was checked after incubation for 24–48 h. The absence of colonies on plates of a treatment was considered as MIC values.

GC-MS Analysis

GC-MS analysis was carried out in an Agilent 6890 gas chromatograph interfaced to an Agilent 5973N mass selective detector MSD (Agilent Technologies, Palo Alto, CA) according to the method suggested by Ramos *et al.* (2012). The GC was equipped with a (5%-phenyl)-methyl polysiloxane HP-5MS column (30 m length × 0.25 mm internal diameter × 0.25 μm film thickness). Helium was used as the carrier gas at a flow rate of 1 mL/min constant flow. The oven temperature was programmed from 45C

(hold for 1 min) to 250C (5 min) at 5C/min in a 47-min total running time. One hundred to four hundred atomic mass unit was used as scanning mass range. Identification of components in EOs was carried out by the comparison of the mass spectra characteristic features with the NIST 05 mass spectral library.

Determination of TPC

TPC was determined by Folin–Ciocalteu assay. Briefly, diluted samples were mixed with Folin–Ciocalteu's reagent (1/10 in distilled water) and left to stand 2–3 min at room temperature. After adding sodium carbonate solution (7.5% w/v) to the mixture, tubes were allowed to stand for 60 min in a dark place at room temperature, then the absorbance of each solution was read against the blank at 765 nm. The same procedure was applied to gallic acid with different concentrations to obtain a calibration curve. Results were expressed as milligram gallic acid equivalents (GAE)/mL (Slinkard and Singleton 1977).

Determination of Total Antioxidants by FRAP Method

FRAP was performed according to Thaipong *et al.* (2006). The fresh working FRAP solution was prepared by mixing 10 mL of acetate buffer (300 mM, pH 3.6), 1 mL of TPTZ (10 mM 2, 4, 6-tripyridyl-striazine) and 1 mL of $FeCl_3.6H_2O$ solution and then warmed at 37C before use. Then, 150 µL of diluted samples was allowed to react in FRAP solution in a 3-mL total volume for 30 min in the dark condition. Readings were then taken at 593 nm and results were expressed as milimol Trolox equivalent per mL.

Determination of Antiradical Activity by DPPH Method

The DPPH radical was determined by the method with some modifications (Ojeda-Sana *et al.* 2013); briefly, 20 µL of each sample in triplicate and six different concentrations and 180 µL of DPPH solution (160 mM) in ethanol was added to a well in a 96-well flat bottom microtitration plate. A DPPH solution was used as blank sample. Plate was incubated for 24 h and absorbance was measured at 515 nm with 10-min interval. The antioxidant activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to the following formula where *Ab* is absorbance of blank sample and *As* is absorbance of a tested sample at the end of the reaction (Delgado Adámez *et al.* 2012; Ramos *et al.* 2012; Ojeda-Sana *et al.* 2013).

$$IC\,(\%) = \left([Ab - As]/Ab\right) \times 100$$

Percentage inhibition after where the reaction gone to completion ("plateau") was plotted against concentration and a linear regression was applied to obtain the IC_{50} value (the concentration of essential oil inhibiting bacterial growth by 50%) (Schwarz *et al.* 2001).

Antimicrobial Effect of EOs in Minced Meat

Sa. typhimurium bacterial suspension was prepared using McFarland standard and further diluted to yield a final concentration of 4 log/g. After inoculation and homogenization in a stomacher of meat samples, thyme and clove EOs were added at MIC × 2 and MIC × 4 values. Afterward, mixtures were homogenized again. Minced beef samples were then divided into 10 g of portion for each sampling time and for each analysis. Samples were kept at 4C and examined during 9 days.

Microbiological Analyses

Microbiological analyses of samples included the determination of the population of *Sa. typhimurium*, aerobic mesophilic microorganism (AMM), psychrotrophic microorganisms, coliform, yeasts and molds. Analyses were carried out at 3-day intervals up to the ninth day at 4C. All of microbial counts were expressed as $\log_{10}$ colony-forming units per g of sample. The media used in the study were obtained from Difco, BD, Dickinson. At each sampling time, 10 g of samples was homogenized in a stomacher bag for 2 min in 90 mL of sterile buffered peptone water (BPW). Serial dilutions were made in BPW and used for enumeration of microorganisms. Brilliant green agar and bismuth sulfite agar were used to evaluate the microbial count of *Sa. typhimurium* after 24 h incubation at 37C. Coliforms were determined using the plate counting method on violet red bile agar as a medium after 24 h of incubation at 37C. AMM was determined on plate count agar (PCA) incubated at 30C for 48 h. Potato dextrose agar was used to determine yeasts and molds after 48 h of incubation at 25C. Determination of psychrotrophic microorganisms was carried out on PCA with 10-day incubation at 4C.

Determination of Lipid Oxidation

Lipid oxidation was measured by the TBA method as described by Djenane *et al.* (2012). Five grams of minced meat samples was taken and mixed well with 10 mL of trichloroacetic acid (10%). Samples were then centrifuged at 10,000 rpm for 30 min at 5C, and supernatants were filtered through quantitative paper. Two milliliters of filtered supernatant was mixed with 2 mL of thiobarbituric acid (20 mM) (99%, Sigma); tube contents were mixed well with vigorous vortexing and incubated at 97C for 20 min in a boiling

water bath. After incubation tubes were cooled immediately and absorbance was measured at 532 nm, the average of three absorbance values was used to determine the oxidative stability.

Color Evaluation

Color was instrumentally measured by L^*, a^* and b^* system using Minolta CR400 (Tokyo, Japan) colorimeter. The instrument was first standardized against a white reference plate. Five measurements were then taken from each sample. The colorimeter directly calculated three color features of L^* (lightness), a^* (red–green component) and b^* (yellow–blue component).

Statistical Analysis

All the analysis were carried out in triplicate and the experimental results obtained were expressed as means ± standard deviation. Statistical analysis of the data was made using analysis of variance (Minitab 16, Minitab Inc., Coventry, UK). Means with a significant difference ($P < 0.05$) were compared using Tukey's test.

RESULTS AND DISCUSSION

GC-MS Analysis of EOs

The chemical compositions of EOs from thyme and clove were determined by comparing the relative retention times and the mass spectra of oil components with mass spectra from data library. The EOs were characterized by one or two dominant components that are listed according to their retention time and percentage contribution (Table 1).

The GC-MS analyses resulted in the identification of 18 components of thyme EO (Table 1). The major components of thyme EO determined by GC-MS were carvacrol (75.27%) followed by cymene (7.84%), thymol (4.51), γ-terpinene (2.96%) and borneol (1.44%). The others comprise terpinene, terpineol, limonene, linalool and eucalyptol (Table 1). There is a great variability and diversity of chemical composition of *Thymus* species due to differences in origin, vegetative cycle and climatic conditions, seasonal and soil variations (Baydar *et al.* 2004; Sokmen *et al.* 2004; Tepe *et al.* 2005; Safaei-Ghomi *et al.* 2009; Verma *et al.* 2010; Ait-Ouazzou *et al.* 2011). Some thymus oils are characterized by the increased percentages of thymol, carvacrol, borneol, linalool or α-terpineol (Tepe *et al.* 2005). The high carvacrol content followed by cymene, thymol, γ-terpinene and borneol, respectively, is in good agreement with the findings of chemical composition of Iran origin thyme oil (Safaei-Ghomi *et al.* 2009). Our findings also showed a good relation with the studies associated with EOs of Turkish origin *Thymus* species (Baydar *et al.* 2004; Sokmen *et al.* 2004). Most of the identified components have also previously been reported as major components of thyme EOs from Tunisian (Hosni *et al.* 2013), Sicilian (Napoli *et al.* 2010) and Greek origins (Economou *et al.* 2011).

TABLE 1. COMPONENTS OF THYME AND CLOVE EOs DETERMINED BY GC-MS ANALYSES

	Compound	Compound R. T. (min)	%
Thyme EO	Carvacrol	17.623	75.27
	Cymene	9.643	7.84
	Thymol	17.248	4.51
	γ-Terpinene	10.632	2.96
	Borneol	13.751	1.44
	β-Bisabolene	22.808	1.33
	α-Terpinene	9.409	1.25
	β-Murcene	8.687	1.10
	4-Terpineol	14.079	0.9
	Caryophyllene	20.649	0.85
	α-Pinene	7.126	0.60
	D-limonene	9.76	0.469
	Linalool	11.827	0.39
	α-Terpinolene	11.488	0.27
	Camphene	7.522	0.25
	α-Phellandrene	9.058	0.15
	Eucalyptol	9.851	0.15
	1-Octen-3-ol	8.378	0.138
Clove EO	Eugenol	19.103	75.20
	Benzyl salicylate	30.78	14.75
	Propylene glycol	3.126	6.02
	β-Caryophyllene	20.653	3.21
	α-Caryophyllene	21.505	0.69
	Caryophyllene oxide	24.642	0.122

EO, essential oil; GC-MS, gas chromatography-mass spectrometry.

Clove EO consists of eugenol (75.2%), benzyl salicylate (14.74%), propylene glycol (6.02%) and also β-caryophyllene (3.21%) (Table 1). Clove EOs have different origins extracted by different methods and have been analyzed by various researchers. Eugenol seems to have the highest proportion of tested clove EOs as it was observed from our results. Besides eugenol, eugenol acetate and β-caryophyllene are the major constituents (Martini *et al.* 1996; Dorman *et al.* 2000; Lee and Shibamoto 2001; Guan *et al.* 2007). GC-MS results differ in the presence and the amount of benzyl salicylate from the literature. The variation in EO composition may be caused by ecological and geographical distribution and also climatic and soil variation condition.

Antimicrobial Activity

Thyme and clove EOs were examined according to their antimicrobial activities using broth microdilution and macrodilution assays. A wide range of concentration of EO

TABLE 2. ANTIMICROBIAL ACTIVITY OF EOs EXPRESSED AS MIC (%) DETERMINED BY BROTH DILUTION ASSAYS

	Thyme EO (% v/v)		Clove EO (% v/v)	
Bacteria	Conc. ranges	MIC	Conc. ranges	MIC
Gram-positive bacteria				
Staphylococcus aureus	2–0.03	0.05	2–0.03	0.5
Carnobacterium divergens	2–0.03	0.06	2–0.03	1
Listeria innocua	2–0.03	0.125	2–0.03	2
Gram-negative bacteria				
Shewanella putrefaciens	2–0.03	0.25	2–0.03	1
Serratia liquefaciens	2–0.03	0.06	2–0.03	0.5
Escherichia coli O157:H7	2–0.03	0.05	2–0.03	0.5
Salmonella typhimurium	2–0.03	0.05	2–0.03	1

EO, essential oil; MIC, minimum inhibitory concentration.

was examined by considering the findings in the literature. Results showed obviously that antimicrobial effects of the plant EOs were concentration dependent for all tested bacteria (Table 2). Twenty-four hours of observation of bacterial growth indicated that the MIC values were 0.05% for all *St. aureus*, *E. coli* O157:H7 and *Sa. typhimurium* for thyme EOs as it is shown in Table 2. *Sh. putrefaciens* was the most resistant bacteria to thyme EOs with a MIC value of 0.25%. In general, food spoilage organisms were observed to be more resistant to thyme EO than foodborne pathogen organisms. *Se. liquefaciens* and *C. divergens* have the MIC value of 0.06% for thyme EO, whereas *L. innocua* was the most resistant one with the MIC value of 0.125% among all the tested bacteria. It was determined that thyme EO was highly effective on gram-negative bacteria except from *Sh. putrefaciens*, and especially foodborne pathogens with a MIC value of 0.05%. Clove bud EO seemed to be effective on both gram-positive and negative bacteria within the range of MIC values between 0.5 and 2.0%. Among the tested bacteria *St. aureus* and *E. coli* O157:H7 were the most sensitive. MIC values of these strains were 0.05 and 0.5% for thyme and clove EO, respectively.

TPC

TPCs of EOs were determined by Folin–Ciocalteu assay. Results were calculated using the equation obtained from the gallic acid calibration curve and expressed as mg GAE/mL. TPC of EOs is presented in Table 3. As it was seen from the table, clove EOs had the highest content of total phenols with 635.327 mg GAE/mL. Thyme EO was seen to be a less rich source of total phenols. Viuda-Martos *et al.* (2010), Wang *et al.* (2008) and Gülçin *et al.* (2004) also demonstrated that clove bud EO had high phenolic content compared with other EOs like thyme EOs (Gülçin *et al.* 2004; Wang *et al.* 2008; Viuda-Martos *et al.* 2010).

Antioxidant Activity by FRAP and DPPH Method

Total antioxidant activities of EOs were determined by both FRAP and DPPH methods (Table 3). Antioxidant activity measured by FRAP method was expressed as millimol Trolox equivalent per mL and calculated using Trolox calibration curve. The ability of EOs was investigated using DPPH assay to observe the ability to act as donors of hydrogen atoms or electrons for the transformation of DPPH into its reduced form DPPH (Ojeda-Sana *et al.* 2013). Results were expressed as 50% of inhibition of DPPH and were represented in Table 3. EOs were able to change the stable violet color of DPPH into yellow-colored DPPH, reaching 50% of reduction with IC_{50} value changing from 0.14 to 9.88 µL/mL. Both results obtained from FRAP and DPPH assays showed nearly the same outcome. Clove EO showed the higher antioxidant activity than thyme EO (Table 3). Some authors also reported that there were differences in the results obtained from these two assays (Gourine *et al.* 2010; Politeo *et al.* 2010). It is difficult to assess the antioxidant activity of a sample referring to a single method as the interpretation of the activity may require a combination of different methods. But it is obvious that it is difficult to

TABLE 3. THE TOTAL PHENOLIC CONTENTS AND ANTIOXIDANT ACTIVITIES OF EOs

Sample	Total phenolic content (GAE mg/mL)	Antioxidant activity	
		FRAP (mmol Trolox/mL)	DPPH (IC_{50} µL/mL)
Thyme	30.5576 ± 8.62	2,150.72 ± 47.03	9.88 ± 0.75
Clove	635.327 ± 11.71	4,357.45 ± 28.83	0.14 ± 0.02

DPPH, 1,1 diphenyl-2-picrylhydrazyl; EO, essential oil; FRAP, ferric-reducing antioxidant power; GAE, gallic acid equivalent.

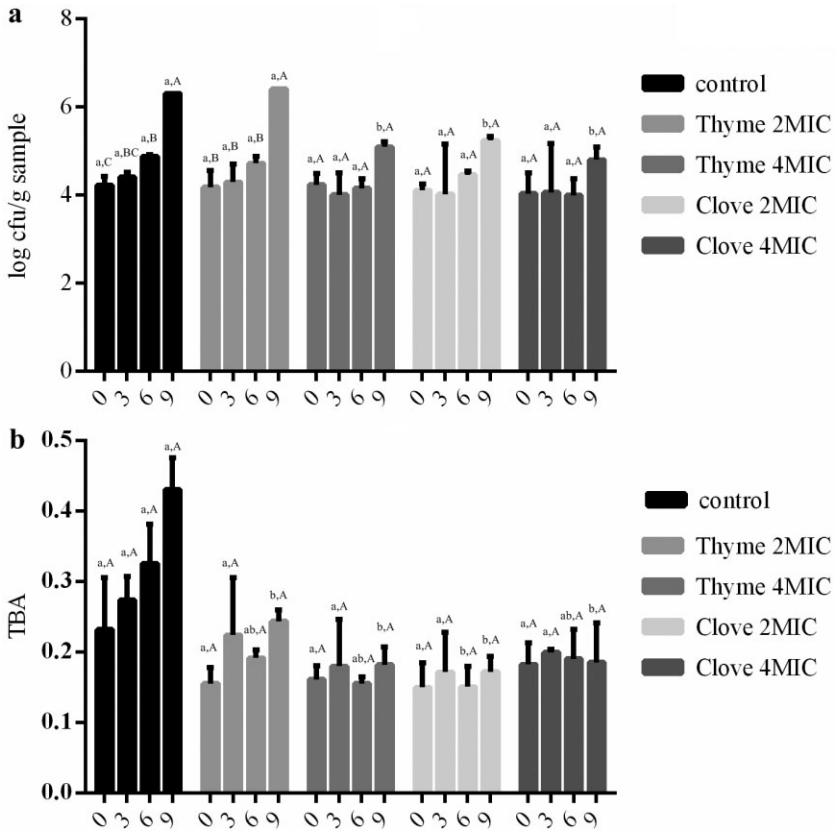

FIG. 1. (a) CHANGE IN *SALMONELLA TYPHIMURIUM* INOCULATED TO GROUND BEEF SAMPLES DURING STORAGE TIME AT +4C
(b) Changes in 2-thiobarbituric acid (TBA) values of minced beef samples during storage at +4C. Data represent means ± standard deviation, $n = 3$. Bars with different letters (a,b) indicate significant difference at $P < 0.05$ within each storage time; A–C indicate significant difference at $P < 0.05$ within each treatment.

compare the results of many different methods as it was shown in this study.

When the relation between TPC and antioxidant activities was compared, phenolic content gives antioxidant activity to clove bud EO.

Antimicrobial Effect of EOs in Minced Beef

The effect of thyme and clove EOs with 2MIC and 4MIC values on the growth of *Sa. typhimurium* in minced meat samples stored at 4C for 9 days was investigated (Fig. 1a). 2MIC and 4MIC values were used as these were below the EO levels, which subsequently not affect the sensorial quality of the meat. Four log cfu/g (low inoculum level) inoculations (Flessa *et al.* 2005; Simpson *et al.* 2008; Petkar *et al.* 2011; Blessington *et al.* 2012) was performed to each sample. Inoculum level can influence survival of bacteria in some foods (Uhart *et al.* 2006, Hayouni *et al.* 2008, Solomakos *et al.* 2008); however, in this study it was not aimed to assess the impact of inoculum level on survival of *Sa. typhimurium* inoculated. The number of the *Sa. typhimurium* did not change significantly during 3 days of storage except a slight increase in control and thyme (2MIC) treated samples (Fig. 1a). At the end of 9-day storage period, significant ($P < 0.05$) difference was detected in the number of *Sa. typhimurium* between control and thyme (4MIC), clove (2MIC and 4MIC) treated samples. When the number of *Sa. typhimurium* increased to 6 log/g in the control sample, the number of the pathogen was ~5 log/g in both thyme (4MIC) and clove (2MIC) treated samples (Fig. 1a). Clove (4MIC) treated sample represented only 0.77 log increase in the number of *Sa. typhimurium* after 9 days. The results indicated that clove (4MIC) EO had more antimicrobial effect ($P < 0.05$) on *Sa. typhimurium* than thyme EO even with 4MIC value.

The effect of thyme and clove EOs with 2MIC and 4MIC values on AMM was also examined during 9 days of storage (Table 4). The minced meat samples showed a high initial AMM as seen in Table 4. The initial AMM was almost the same for all samples approximately 7 log cfu/g, indicating high contamination level for aerobic plate count for fresh meat (ICMSF 1986). During the time periods AMM showed an increase of up to 11 log/g except the clove EO (4MIC) treated sample. At the end of storage for 9 days, AMM increased up to 10 log cfu/g for clove EO (4MIC) treated sample. There was a 1 log cycle difference between the control and other treated samples and clove (4MIC) treated sample (Table 4).

Coliform was also determined for control- and EO-treated samples (Table 4). The initial TCC was almost the same for all groups of samples (Table 4). During the

TABLE 4. CHANGE IN AEROBIC MESOPHILIC COUNT OF GROUND BEEF SAMPLES DURING STORAGE AT 4C

		Storage time (days)			
	Samples	0	3	6	9
Aerobic mesophiles (log cfu/g)	Control	$7.28 \pm 0.11^{a,B}$	$8.43 \pm 0.29^{a,B}$	$9.44 \pm 0.62^{a,AB}$	$11.72 \pm 0.66^{a,A}$
	Thyme–2MIC	$7.19 \pm 0.03^{a,C}$	$8.16 \pm 0.03^{a,BC}$	$9.60 \pm 0.31^{a,B}$	$11.86 \pm 0.53^{a,A}$
	Thyme–4MIC	$7.18 \pm 0.15^{a,C}$	$8.43 \pm 0.10^{a,BC}$	$9.19 \pm 0.21^{a,B}$	$11.18 \pm 0.47^{a,A}$
	Clove–2MIC	$7.16 \pm 0.14^{a,D}$	$8.26 \pm 0.19^{a,C}$	$9.24 \pm 0.11^{a,B}$	$11.34 \pm 0.14^{a,A}$
	Clove–4MIC	$7.09 \pm 0.06^{a,C}$	$8.07 \pm 0.41^{a,BC}$	$9.09 \pm 0.05^{a,AB}$	$10.02 \pm 0.33^{a,A}$
Coliform (log cfu/g)	Control	$4.41 \pm 0.04^{a,A}$	$4.65 \pm 0.49^{a,A}$	$5.01 \pm 0.34^{a,A}$	$6.07 \pm 0.19^{a,A}$
	Thyme–2MIC	$4.56 \pm 0.08^{a,A}$	$4.74 \pm 0.06^{a,A}$	$4.79 \pm 0.44^{a,A}$	$5.89 \pm 0.21^{a,A}$
	Thyme–4MIC	$4.50 \pm 0.25^{a,A}$	$4.28 \pm 0.03^{a,A}$	$4.74 \pm 0.43^{a,A}$	$5.69 \pm 0.26^{a,A}$
	Clove–2MIC	$4.42 \pm 0.26^{a,A}$	$4.62 \pm 0.12^{a,A}$	$4.59 \pm 0.63^{a,A}$	$5.32 \pm 0.30^{ab,A}$
	Clove–4MIC	$4.36 \pm 0.35^{a,A}$	$4.57 \pm 0.18^{a,A}$	$4.39 \pm 0.17^{a,A}$	$4.10 \pm 0.46^{b,A}$
Yeast and molds (log cfu/g)	Control	$6.30 \pm 0.43^{a,C}$	$7.71 \pm 0.23^{a,BC}$	$9.29 \pm 0.00^{ab,B}$	$11.63 \pm 0.43^{a,A}$
	Thyme–2MIC	$6.23 \pm 0.35^{a,C}$	$7.31 \pm 0.11^{a,C}$	$9.41 \pm 0.00^{a,B}$	$11.82 \pm 0.45^{a,A}$
	Thyme–4MIC	$6.06 \pm 0.36^{a,C}$	$7.70 \pm 0.27^{a,BC}$	$9.18 \pm 0.00^{ab,B}$	$11.22 \pm 0.53^{a,A}$
	Clove–2MIC	$6.04 \pm 0.19^{a,D}$	$7.50 \pm 0.25^{a,C}$	$9.22 \pm 0.00^{ab,B}$	$11.35 \pm 0.00^{a,A}$
	Clove–4MIC	$6.42 \pm 0.09^{a,C}$	$7.33 \pm 0.21^{a,C}$	$8.81 \pm 0.25^{b,B}$	$10.09 \pm 0.11^{a,A}$
Psychrotrophs (log cfu/g)	Control	$7.05 \pm 0.01^{a,C}$	$8.01 \pm 0.23^{a,BC}$	$8.95 \pm 0.00^{c,B}$	$11.64 \pm 0.58^{a,A}$
	Thyme–2MIC	$6.87 \pm 0.18^{a,C}$	$7.54 \pm 0.12^{a,C}$	$9.24 \pm 0.00^{b,B}$	$11.85 \pm 0.44^{a,A}$
	Thyme–4MIC	$6.99 \pm 0.02^{a,C}$	$7.89 \pm 0.21^{a,BC}$	$8.92 \pm 0.00^{c,B}$	$11.27 \pm 0.42^{a,A}$
	Clove–2MIC	$7.02 \pm 0.08^{a,C}$	$7.65 \pm 0.24^{a,C}$	$9.20 \pm 0.00^{b,B}$	$11.38 \pm 0.21^{a,A}$
	Clove–4MIC	$7.01 \pm 0.01^{a,B}$	$7.74 \pm 0.48^{a,B}$	$9.32 \pm 0.03^{a,A}$	$10.15 \pm 0.34^{a,A}$

[a–c] Different letters indicate significant differences ($P < 0.05$) within each storage time.
[A–D] Different letters indicate significant differences ($P < 0.05$) within each treatment.
cfu, colony-forming units; MIC, minimum inhibitory concentration.

storage period TCC increased for control, thyme (2MIC and 4MIC) and clove (2MIC) treated samples. However, a 0.26 log cycle reduction was observed for the clove (4MIC) treated sample at the end of 9 days. The results of TCC indicated that clove EO inhibited the growth of coliform bacteria during 9-day period (Table 4).

The initial numbers of yeasts and molds were almost the same with approximately 6 log/g for all groups of samples. The number of yeasts and molds of control and treated samples increased dramatically during 9 days of storage (Table 4). Although the load of all samples exceeded to 11 log/g at the end of 9 days, clove (4MIC) treated sample increased up to 10.09 log/g (Table 4). There was no great difference between the number of yeasts and molds found in samples.

A dramatic growth of psychrotrophic organisms was observed during 9 days of storage for all groups of samples (Table 4). Counting of mesophilic bacteria in ground beef is the useful tool for determining post-processing contamination level, monitoring microbial quality and shelf life evaluation; however, the most prominent microbial group responsible for the spoilage of ground beef as seen also in our results is the psychrotrophs. There was no significant difference between the number of psychrotrophs in the samples. However, control, thyme (2MIC and 4MIC) and clove (2MIC) EO-treated samples showed growth ended up with ~11 log/g, whereas clove (4MIC) ended up with a ~10 log/g of psychrotrophic organisms (Table 4).

Our findings represented that clove EO restricted the growth of *Sa. typhimurium* and coliform bacteria comparing with thyme EO with 4MIC value. It was not seemed to be inhibiting the aerobic mesophiles, yeasts and molds and also psychrotrophic microorganisms. This finding confirms the statement of the thyme, and clove EOs have significant bacteriostatic/inhibiting properties for pathogenic and spoilage microorganisms and as a result they were less effective as food preservative in food (Bensid *et al.* 2014). However, their growth was lesser than the control sample.

Effect of EOs on Lipid Oxidation in Minced Beef

The oxidation conditions of minced meat samples were evaluated by observing the absorbance at 532 nm. TBA values were determined using the thiobarbituric acid reactive substances assay (Fig. 1b). Measurements were obtained over 9 days of storage, and TBA values of control showed a rapid increase with increasing storage time. TBA values of all treatments on day 0 were significantly lower ($P < 0.05$) than those for the control sample (Fig. 1b). Moreover, treatment samples had significantly lower ($P < 0.05$) TBA values than control at each day of testing throughout sixth and ninth days of the storage period. Although there was no significant difference among treated samples, each treatment showed significant reduction ($P < 0.05$) comparing with

TABLE 5. CHANGES IN COLOR OF MINCED BEEF SAMPLES DURING STORAGE AT 4C

Samples		Storage time (days) 0	3	6	9
Control (with inoculum)	*L**	49.57 ± 0.28^{a,A}	50.03 ± 0.18^{a,A}	50.89 ± 0.15^{a,A}	50.79 ± 1.64^{a,A}
	*a**	19.77 ± 1.53^{a,A}	18.02 ± 0.62^{a,A}	16.70 ± 0.21^{a,A}	14.81 ± 1.21^{a,A}
	*b**	10.09 ± 0.09^{a,A}	10.40 ± 0.07^{a,A}	10.49 ± 0.4^{a,A}	9.44 ± 0.57^{a,A}
Thyme–2MIC	*L**	49.91 ± 0.45^{a,A}	49.85 ± 0.46^{a,A}	49.23 ± 0.07^{a,A}	51.19 ± 0.62^{a,A}
	*a**	19.61 ± 2.26^{a,A}	16.26 ± 5.08^{a,A}	16.76 ± 0.91^{a,A}	16.54 ± 3.65^{a,A}
	*b**	9.06 ± 0.85^{a,A}	10.21 ± 1.74^{a,A}	10.64 ± 0.57^{a,A}	10.46 ± 0.43^{a,A}
Thyme–4MIC	*L**	49.82 ± 0.22^{a,A}	50.29 ± 0.16^{a,A}	50.24 ± 1.27^{a,A}	49.08 ± 1.79^{a,A}
	*a**	19.60 ± 0.74^{a,A}	16.88 ± 0.69^{a,A}	18.83 ± 1.34^{a,A}	18.50 ± 0.96^{a,A}
	*b**	9.72 ± 0.18^{a,A}	10.73 ± 0.32^{a,A}	10.70 ± 0.47^{a,A}	9.99 ± 0.68^{a,A}
Clove–2MIC	*L**	49.35 ± 0.29^{a,A}	50.96 ± 1.32^{a,A}	50.36 ± 0.64^{a,A}	51.00 ± 0.74^{a,A}
	*a**	20.80 ± 0.90^{a,A}	17.56 ± 1.04^{a,A}	18.57 ± 0.99^{a,A}	18.44 ± 0.99^{a,A}
	*b**	9.89 ± 0.30^{a,B}	10.69 ± 0.12^{a,AB}	10.95 ± 0.12^{a,A}	11.35 ± 0.12^{a,A}
Clove–4MIC	*L**	48.65 ± 1.25^{a,A}	50.60 ± 1.35^{a,A}	49.63 ± 0.82^{a,A}	51.12 ± 0.62^{a,A}
	*a**	18.29 ± 0.83^{a,A}	19.38 ± 0.45^{a,A}	19.24 ± 2.32^{a,A}	17.60 ± 4.45^{a,A}
	*b**	9.37 ± 0.01^{a,A}	10.48 ± 0.66^{a,A}	11.28 ± 0.17^{a,A}	11.09 ± 0.58^{a,A}

The values were expressed as mean ± standard deviation, $n = 3$.

^{A,B} Different letters indicate significant differences ($P < 0.05$) within each treatment.

$^{a-b}$ Means having different letters indicate significant difference at $p < 0.05$ within each storage time.

MIC, minimum inhibitory concentration.

control (Fig. 1b). The results indicated that thyme (2MIC and 4MIC) and clove (2MIC and 4MIC) EOs retarded lipid oxidation during 9 days of storage at 4C. Although clove EO showed the higher *in vitro* antioxidant activity than thyme EO (Table 3) in minced meat application, both of the EO treatments showed significant reduction ($P < 0.05$) in TBA value comparing with control. Lipid oxidation in food products is considered to be one of the important factors limiting product quality and acceptability due to the production of potentially toxic reactive oxygen species and off-flavors from unsaturated fatty acids (Cui *et al.* 2012). Antioxidant activity of various EOs and components has been determined using TBA assay, which is an index of secondary lipid oxidation measuring malondialdehyde in both model and real meat systems, showing a potential for protection of meat from oxidation (Guillén-Sans and Guzmán-Chozas 1998; Ruberto and Baratta 1999; Tanabe *et al.* 2002; Fasseas *et al.* 2007; Bensid *et al.* 2014). Protective effectiveness of EOs on meat oxidation stability was also shown in this study (Fig. 1b), which are in agreement with those of previous findings.

Effect of EOs on Color in Minced Beef

The objective color parameters L^*, a^* and b^* during storage according to different treatments evaluated are shown in Table 5. As seen in the lightness there was no significant darkening or browning in color due to metmyoglobin formation between all group of samples in terms of both EO treatments and storage time (Table 5). The initial L^* values were maintained during the storage period. a^* value of control sample showed a dramatic reduction following the time period of storage. There was no significant difference among a^* values of control and EO-treated samples throughout the storage period (Table 5). However, a^* value was maintained during 9 days of storage in clove EO (4MIC) treated samples comparing with control sample. There was also no significant difference between b^* values of treated samples (Table 5). But b^* values showed a significant ($P < 0.05$) alteration over the time period (Table 5). The color parameters lightness, redness and yellowness were not significantly affected when compared with control by the addition of EOs; this fact suggests that no characteristic color alteration means no rejection from customers.

CONCLUSION

The biological activities of natural compounds such as EOs are highly affected by their chemical compositions. EOs are complex natural mixtures that behind their major and minor components are also effective on the biological activities, possibly by producing a synergistic effect between other components. EOs are complex mixtures. Biological activities are affected by minor components possibly by producing a synergistic effect, beside the major ones. Results showed that thyme and clove EOs showed strong antimicrobial activity against all the tested spoilage and pathogenic bacteria with different MIC values. Therefore, EOs could have potential applications in meat industries. Although thyme and clove EOs restricted the growth of

Sa. typhimurium and *Coliform* bacteria, it was not seemed to be inhibiting the aerobic mesophiles, yeasts and molds and also psychrotrophic microorganisms in ground beef application study. As it was demonstrated in the results, EOs were not effective negatively on color parameters. Thus, both L^* and a^* values were maintained during the refrigeration storage period. This study showed that the most prominent and featured effect of EOs was the antioxidant capacity in the ground beef meat application study.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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