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Effect of *Thymus vulgaris* and *Bunium persicum* essential oils on the oxidative stability of virgin olive oil

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SUMMARY: Natural antioxidants are becoming a major focus because natural food ingredients are safer than synthetic types. The aim of this study was to investigate the protective effects of *Thymus vulgaris* and *Bunium persicum* essential oils (EO) on the oxidation of virgin olive oil (VOO) during accelerated storage. The antioxidant activities of EOs were compared with those of α-tocopherol and BHT. GC/MS analyses revealed that thymol (28.50%), *p*-cymene (27.14%), carvacrol (18.36%), and γ-terpinene (4.97%) are the main components of *T. vulgaris* EO, while cuminaldehyde (32.81%), γ-terpinene (16.02%) and *p*-cymene (14.07%) are the main components of *B. persicum* EO. Both EOs provided protection for the VOO, inhibiting the formation of primary and secondary oxidation products although *T. vulgaris* EO showed greater protection against the oxidation process than *B. persicum* EO. The effect of *T. vulgaris* essential oil on the oxidation inhibition of VOO was similar to that of BHT. α-Tocopherol showed no measurable effect on improving the oxidative stability of VOO. This study suggests that *T. vulgaris* and *B. persicum* EOs can be used to improve the oxidative stability of VOO.

***KEYWORDS*:** Bunium persicum*; Oxidation;* Thymus vulgaris*; Tocopherol; Virgin olive oil*

RESUMEN: ***Efecto de aceites esenciales de tomillo y comino negro sobre la estabilidad oxidativa de aceites de oliva virgen***. En los antioxidantes naturales se está centrando actualmente más la atención dado que los ingredientes naturales son más seguros que los sintéticos. El objetivo de este estudio fue estudiar el efecto protector de aceites esenciales (AE) de *tomillo* y *comino negro* en la oxidación del aceite de oliva virgen (AOV) durante un almacenamiento acelerado. Las actividades antioxidantes de los AE se compararon con las del α-tocoferol y BHT. Los análisis de GC/MS mostraron que timol (28,50%), *p*-cimeno (27,14%), carvacrol (18,36%), y γ-terpineno (4,97%) son los principales componentes de AE de *tomillo*, mientras que cuminaldehido (32,81%), γ-terpineno (16,02%) y *p*-cimeno (14,07%) lo son de AE de *comino negro*. Ambos AE proporcionan protección al AOV, inhibiendo la formación de productos de oxidación primarios y secundarios. AE de *tomillo* mostró mayor efecto contra la oxidación que el AE de *comino negro*. El efecto del AE de *tomillo* en la inhibición de la oxidación de VOO fue similar al del BHT. El α-tocoferol no mostró ningún efecto sobre la mejora de la estabilidad oxidativa del AOV. Este estudio sugiere que los AE de *tomillo* y *comino negro* se pueden utilizar para mejorar la estabilidad oxidativa del AOV.

***PALABRAS CLAVE*:** *Aceite de oliva virgen; Comino negro; Oxidación; Tocoferol; Tomillo*

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1. INTRODUCTION

Virgin olive oil (VOO) is highly appreciated for its delicious taste and aroma, as well as for its nutritional properties. Its nutritional benefits are primarily related to its fatty acid composition, mainly due to the high content of oleic acid and also to the balanced ratio of saturated and polyunsaturated fatty acids (PUFAs). Furthermore, olive oil presents considerable amounts of natural antioxidants (Moldão-Martins *et al*., 2004). During storage, oxidation reactions reduce the high nutritional value of VOO and modify its characteristic flavor through the development of off – flavors derived from hydro peroxide decomposition products (Morales *et al*., 1997).

Among the most usual strategies to inhibit lipid oxidation in vegetable oils, the addition of antioxidants has been practiced for decades. However, recent studies claim that synthetic antioxidant compounds could pose possible hazards and carcinogenic effects (Sasaki *et al*., 2002). Moreover, according to the Codex Alimentarius Commission, synthetic antioxidants are not permitted for use in VOO (Codex, 2011). Furthermore, natural antioxidants such as tocopherols and their derivatives, which can be used as alternatives to BHA and BHT, exhibit little effectiveness in some systems and also increase manufacturing costs. Consequently, there is the need to identify alternative natural and safe sources of antioxidants to be incorporated into food products. These safer sources of antioxidants can be especially of plant origin, where relevant research has notably increased in recent years.

Buniumpersicum is an important aromatic plant that belongs to the Apiaceae family. It originates from central Asia to North India. The γ-Terpinene, cuminaldehyde, *p*-cymene and limonene are major components of *B. persicum* EO (Mazidi *et al*., 2012). *Thymus vulgaris* (also known as common thyme) is a member of the Labiatae family. Dried plant materials of *T. vulgaris* contain 1 - 2.5% EO. Meanwhile, Thymol, carvacrol, *p*-cymene, and γ-terpinene are the main components of *T. vulgaris* EO (Golmakani and Rezaei, 2008).

Essential oils (EOs) obtained from aromatic plants have received considerable attention in the current era of concerns for food safety. For instance, a report claims that *Carumcopticum* EO (0.075%) is more effective than BHA and BHT (0.02%) in retarding the oxidation of sunflower oil (Hashemi *et al*., 2014). Furthermore, Inanc and Maskan (2014) reported that carvacrol can significantly improve the oxidative stability of palm oil in comparison with the control sample. Also, the antioxidant activity of carvacrol is known to be similar to that of BHT. Ruberto and Baratta, (2000) reported that *γ*-terpinene (which is the major component of *B. persicum* EO) shows a very high antioxidant activity. More specifically, γ-terpinene has a comparable activity to that of α-tocopherol.

The objective of this study is to compare the effects of *T. vulgaris* and *B. persicum* EOs on the oxidation of VOO during accelerated storage. The antioxidant activities of EOs are compared with those of α-tocopherol and BHT.

2. MATERIALS AND METHODS

2.1. Materials

Dried seeds of *B. persicum* and the dried aerial parts of *T. vulgaris* were purchased from a local market in Shiraz, Iran. The genus and species of both plants were confirmed by experts from the Herbarium of Biology Department at Shiraz University, Shiraz, Iran. VOO was supplied from the Etka Oil Company (Rudbar, Iran). All chemicals used in this research were of analytical grade and were purchased from Merck (Darmstad, Germany) and Sigma–Aldrich (St. Louis, MO, USA).

2.2. Extraction of EO

Fifty grams of each plant sample were mixed with 500 mL of distilled water. They were hydrodistillated for 3 h using a Clevenger-type apparatus (Golmakani and Rezaei, 2008). The final yields of *T. vulgaris* and *B. persicum* EOs were reported here to be 2.13±0.04% and 1.92±0.32, respectively. EO samples were dried over anhydrous sodium sulphate and stored in sealed vials at -18 °C until further use.

2.3. GC Analysis of EO

The identification of EO constituents, known as a qualitative analysis, was made using a GC (7890A, Agilent Technologies, Santa Clara, CA) which was coupled with a mass spectrometer (5975C, Agilent Technologies, Santa Clara, CA) operating at 70 eV ionization energy, 0.5 s/scan, and a mass range of 35–400 atomic mass units (amu), equipped with a HP-5MS capillary column (5% Phenyl Polysilphenylene-siloxane; 30 m length; and 0.25 mm internal diameter; 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA). One μL of the EO sample was injected into the GC/MS in split mode (split ratio: 1/100). Helium was used as the carrier gas with a flow rate of 0.9 mL/min. The injector and detector temperatures were at 280 °C. The oven temperature was programmed to start at 60 °C and gradually heated up to a temperature of 210 °C at a rate of 3 °C/min. Thereafter, the rate of temperature elevation was such that the temperature increased by 20 °C/min until the point of 240 °C was reached, whereupon the temperature was held constant for 8.5 min. The MSD ChemStation Software (G1701EA, E.02.01.1177, Agilent Technologies, Santa Clara, CA) was employed to analyze the mass spectra and chromatograms. The compounds were

identified by comparing their mass spectral fragmentation patterns with those stored in the data bank (Wiley/NBS) and with mass spectral data derived from the relevant literature (Golmakani and Rezaei, 2008; Hashemi *et al*., 2014; Mazidi *et al*., 2012; Moldão-Martins *et al*., 2004; Shahsavari *et al*., 2008; Zeng *et al*., 2011). In addition, a quantitative analysis of EO constituents was made under the same chromatographic conditions using a GC, coupled with a flame ionization detector (FID). The relative data for percentages were obtained from the electronic integration of chromatogram peak areas.

2.4. Determination of EO antioxidant activity

The antioxidant activities of the EOs were evaluated based on the free radical scavenging capacity and their reducing power.

2.4.1. Free radical scavenging capacity

The free radical scavenging capacity of the EO samples were measured using DPPH° (2,2-diphenyl-1-picrylhydrazyl radical) as described by Mazidi *et al*. (2012). The IC_{50} value is defined as the concentration of the antioxidant which is required to inhibit 50% of the DPPH° activity. Here, the IC_{50} value was determined through graph plotting, by considering the percentage of the remaining DPPH° against the EO concentrations.

2.4.2. Ferric reducing assay

The ferric reducing power of EOs and that of the positive control (L-ascorbic acid) were determined here according to the method of Ardestani and Yazdanparast (2007). The reducing power was measured by reducing the Fe (III) to Fe (II). One mL of each EO solution (100–10000 $mg{\cdot}L^{-1}$) was mixed with 2.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 10 $g{\cdot}L^{-1}$ potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated at 50 °C for 20 min, whereupon 2.5 mL of 100 $g{\cdot}L^{-1}$ trichloro acetic acid was added to the mixture and centrifuged for 10 min at 3000 g. The upper layer of solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (1 $g{\cdot}L^{-1}$). The absorbance was measured by the spectrophotometer at 700 nm. Generally, a higher absorbance value indicates a higher reducing power. Results were expressed as mg ascorbic acid equivalents per gram of sample.

2.4.3. Cupric ion reducing assay

The cupric ion reducing power of the EOs and the positive control (L-ascorbic acid) were determined here in a test tube by mixing together 1 mL of $CuCl_2$ solution (10 mM), 1 mL neocuproinemethanolic solution (7.5 mM) and 1 mL ammonium acetate aqueous buffer solution (1 M). EO sample solutions (0.50 mL, 100–10000 $mg{\cdot}L^{-1}$) and H_2O (0.60 mL) were added to the initial mixture so that the final volume reaches 4.10 mL. The tubes were stoppered and, after 30 min, the absorbance was recorded against the blank at 450 nm (Apak *et al*., 2008). Results are expressed as mg of ascorbic acid equivalents per gram of sample.

2.5. Determination of initial characteristics of VOO

The initial characteristics of VOO were determined by measuring its chemical and physical properties as follows.

2.5.1. Determination of free fatty acid content

Free fatty acid content was determined according to the AOCS official method (Cd 3d-63) and was reported as a percentage of oleic acid (AOCS, 2000).

2.5.2. Determination of fatty acid composition of VOO

Fatty acid methyl esters were prepared here according to the method described by Golmakani *et al*. (2012). The composition and types of fatty acids in the VOO sample was analyzed using a GC system (SP-3420A, Beijing Beifen-Ruili Analytical Instrument, Beijing, China) which is a device equipped with a split/splitless injector, a flame ionization detector (FID) and a BPX70 capillary column (Bis–cyanopropylsiloxane-silphenylene, 120 m × 0.25 mm internal diameter; 0.25 μm film thickness, *SGE* Analytical Science, Melbourne, *Australia*). The temperatures of the column, injector and detector were set at 198 (isothermal), 250 and 300 °C, respectively. Nitrogen was used as the carrier gas. One μL of fatty acid methyl esters was then injected into the column with a split ratio of 1:10 accordingly. The fatty acids in the VOO were identified according to the retention times for standard fatty acids injected under the same operating conditions. The quantities of fatty acids were measured by calculating their relative peak areas.

2.5.3. Determination of total phenolic content

Here, total phenols were isolated from a solution of oil in hexane by triple-extraction with water–methanol (60:40 v/v). The amounts of phenols were estimated using the Folin–Ciocalteu reagent at 725 nm. Results were expressed as mg of gallic acid per grams of VOO (Casal *et al*., 2010).

2.5.4. Determination of chlorophyll and carotenoid contents

Chlorophyll and carotenoid contents were determined at 470 and 670 nm, respectively, according to the method described by Minguez-Mosquera *et al*. (1990).

2.5.5. Determination of oxidation indices of VOO

The peroxide value (PV) was determined according to the AOCS official method (Cd 8–53) and was expressed as meq $O_2 \cdot kg^{-1}$ VOO. The *p*-Anisidine value (AV) was determined using the AOCS official method (Cd 8–53) and was expressed as $mg \cdot kg^{-1}$ VOO (AOCS, 2000). The TOTOX value (total oxidation value; AV + 2 PV) is used as an empirical measure of the relevant precursors, the non-volatile carbonyls, present in the processed oils. The TOTOX value can also be used as a measure of any further oxidation products developed after storage (Frankel, 2012). The K_{232} and K_{268} extinction coefficients were determined according to the AOCS official method (ch 5–91) by measuring the absorbance of a pertinent solution (1% concentration) in isooctane at 232 and 268 nm, with 1 cm of pass length (AOCS, 2000).

2.6. Accelerated storage of VOO

Here, the EOs of *T. vulgaris and B. persicum* were added to the VOO at a concentration of 1000 $mg \cdot L^{-1}$. The BHT and α-tocopherol at 100 $mg \cdot L^{-1}$ concentration are added to the VOO. For the control group, a sample with no added antioxidants was used. VOO samples (70 mL) were then kept in open amber bottles in an incubator at 70±1 °C for 42 days. The PV, AV, K_{232} and K_{268} were measured weekly. Also, the chlorophyll and carotenoids were measured every two weeks, as they have been in this research.

The induction period of PV (IP_{pv}) is commonly considered as the number of days required for a sample to reach a PV of 20 meq $O_2 \cdot kg^{-1}$, which is beyond the maximum permitted limit, when a samlpe consequently loses the classification of VOO category (Hashemi *et al*., 2014; IOC, 2015).

Generally, the IP_{K232} and IP_{K268} are considered as the number of days required to reach the upper legal limits of K_{232} and K_{268} (with a K_{232} value of 2.6 and K_{268} value of 0.25). These are established by the International Olive Council (IOC) for VOO (IOC, 2015).

The Average percentage of difference was calculated according to eq. (1)

$$\text{Average percent difference} = 100 \times \frac{IP_{PV} - IP_K}{IP_{PV}} \qquad \text{eq. (1)}$$

The effectiveness of the antioxidant, also known as the stabilizing effect, is defined as the induction period extension (IPE) according to eq. (2) (Abramovic and Abram, 2006).

$$IPE\ (\%) = \frac{IP_a - IP_c}{IP_c} \times 100 \qquad \text{eq. (2)}$$

2.7. Statistical analysis

All experiments were performed in triplicate and the data were reported as mean values of the measurements while presenting the standard deviation values in tables and the standard deviation bars in figures. A general linear model (GLM) procedure from SAS (Statistical Analysis Software, version 9.1; SAS Institute Inc. Cary, NC) was used for the comparison of mean values. The simple regression equations for the chemical variables that were obtained from the storage study of VOO (PV, K_{232}, and K_{268}) were calculated by Microsoft Office Excel 2010.

3. RESULTS AND DISCUSSON

3.1. GC analysis of EO

The chemical compositions of *T. vulgaris* and *B. persicum* EOs are presented in Table 1. The total numbers of chemical constituents identified in the EOs were measured to be 24 for *B. persicum* and 29 for *T. vulgaris* EO. The main components of *B. persicum* EO were cuminaldehyde (32.81%) and *monoterpene hydrocarbons* (γ-terpinene (16.02%) and *p*-cymene (14.07%)). Previous reports suggest that the antioxidant activity of γ-terpinene is significantly higher than that of *p*-cymene (Ruberto and Baratta, 2000). The *T. vulgaris* EO was characterized mainly by monoterpene phenols (thymol (28.50%) and carvacrol (18.36%)) and also by their corresponding monoterpene hydrocarbon precursors (*p*-cymene (27.14%) and γ-terpinene (4.97%)). Similarly, Golmakani and Rezaei (2008) reported that thymol, carvacrol, *p*-cymene, and γ-terpinene were the major compounds of *T. vulgaris* EO. On the contrary, camphor was not detected in our samples, but had been found as the main component of *T. vulgaris* EO from the plant source in Eastern Morocco (Imelouane *et al*., 2009). According to Ruberto and Baratta (2000) thymol and carvacrol possessed stronger antioxidant activity than camphor.

3.2. Determination of antioxidant activity of EOs

Antioxidants can scavenge radical species by hydrogen donation, which causes a decrease in DPPH° absorbance at 517 nm (Zeng *et al*., 2011). The radical scavenging capacity of *T. vulgaris and B. persicum* EOs are presented in Table 2. Both EOs managed to reduce the stable, purple-colored radical DPPH° into yellow-colored DPPH–H, reaching IC_{50} values of 4.15 $mg \cdot mL^{-1}$ for *B. persicum* EO and 0.50 $mg \cdot mL^{-1}$ for *T. vulgaris* EO. There was no significant difference between the IC_{50} value of *T. vulgaris* EO with that of BHT, whereas the IC_{50} value of *B. persicum* EO was significantly higher than BHT. However, in previous studies, the radical scavenging capacity of *B. persicum* EO (IC_{50} value

TABLE 1. Chemical compositions (%) of *Thymus vulgaris* and *Bunium persicum* essential oils

		Relative peak area (%)	
Compound	**Retention index**	***Bunium persicum***	***Thymus vulgaris***
Monoterpene hydrocarbons			
α-Thujene	925	0.23	0.33
α-Pinene	932	1.52	1.74
Camphene	946	0.40	1.37
β-Pinene	975	2.72	0.18
Myrcene	989	ND[a]	1.17
β-Myrcene	990	ND	ND
α-Phellandrene	1004	ND	0.32
α-Terpinene	1015	0.39	1.18
p-Cymene	1029	14.07	27.14
Limonene	1030	0.16	0.17
γ-Terpinene	1058	16.02	4.97
Meta-Cymenene	1087	ND	0.19
α-Terpinolene	1095	0.41	ND
Cuminyl	1304	4.43	ND
2-Thujene	1470	1.65	ND
Oxygenated monoterpenes			
Cis-Sabinene hydrate	1065	ND	0.11
Trans-Linalool oxide	1070	ND	0.11
Linalool	1102	ND	3.60
Borneol	1164	0.53	3.29
Terpinene-4-ol	1175	0.50	1.43
α-Terpineol	1189	ND	0.08
Cuminaldehyde	1257	32.81	ND
Thymol	1291	ND	28.50
2-Caren-10-al	1297	4.28	ND
Carvacrol	1313	0.51	18.36
p-Mentha-1,4-diene 7-ol	1337	8.67	ND
Piperitenone	1343	ND	0.12
Thymol acetate	1354	ND	0.54
Carvacrol acetate	1372	ND	0.33
Cuminyl acetate	1434	0.58	ND
Sesquiterpene hydrocarbons			
(E)-Caryophyllene	1418	0.86	1.76
Trans-Caryophyllene	1422	0.64	ND
Aromadendrene	1437	ND	0.18
α-Humulene	1451	ND	0.40
Oxygenated Sesquiterpenes			
Spathulenol	1576	ND	0.55
Caryophyllene oxide	1582	0.92	1.42
Caryophylla-4(14),8(15)-dien-5-b-ol	1633	ND	0.32
7-epi-a-Eudesmol	1658	ND	0.12
α-Bisabolol	1686	0.32	ND
Other compounds			
Elemicin	1563	1.25	ND
Butane, 1,2,3,4-tetrachlorohexafluoro	1668	5.92	ND

TABLE 1 (*Continued*)

Compound	Retention index	Relative peak area (%)	
		Bunium persicum	***Thymus vulgaris***
Monoterpene hydrocarbons		42.00	38.76
Oxygenated monoterpenes		47.88	56.47
Sesquiterpene hydrocarbons		1.50	2.34
Oxygenated Sesquiterpenes		1.24	2.41
Total identified compounds (%)		99.79	99.98

[a]Not detected.

TABLE 2. Radical scavenging capacity and reducing power of *Thymus vulgaris* and *Bunium persicum* essential oils (EOs)

Antioxidant parameter	BHT	*B. persicum* EO	*T. vulgaris* EO
IC_{50} (mg mL^{-1})	0.20±0.03[b]	4.15±0.23[a]	0.50±0.06[b]
Ferric reducing power (mg ascorbic acid equivalents g EO^{-1})	-	457.32± 8.63[b]	701.22±8.61[a]
Cupric ion reducing power (mg ascorbic acid equivalents g EO^{-1})	-	520.46±3.21[b]	720.46±3.22[a]

Values given are the means of three replicates ± standard deviation.
In each row, means with different letters are significantly different ($p < 0.05$).

of 0.88 mg mL^{-1}) was significantly higher than that of *T. vulgaris* EO (IC_{50} value of 8.9 mg·mL^{-1}) and lower than that of BHT and α-tocopherol (IC_{50} values of 0.58 and 0.2 mg·mL^{-1}, respectively) (Fazel *et al*., 2007; Shahsavari *et al*., 2008; Zeng *et al*., 2011).

Ferric ion (Fe^{3+}) and cupric reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties (Apak *et al*., 2008; Zhang *et al*., 2010). The reducing powers of *T. vulgaris* and *B. persicum* EOs are presented in Table 2. Both results obtained from ferric and cupric reduction assays showed nearly the same outcome. *T. vulgaris* and *B. persicum* EOs showed some degree of hydrogen-donating capacity, but the capacities were, as expected, inferior to ascorbic acid. This is in agreement with the radical scavenging capacity results that *T. vulgaris* EO showed stronger reducing power than *B. persicum* EO. These results indicate that EOs rich in phenolic monoterpenes are more potent reductants and radical scavengers than those rich in monoterpene hydrocarbons.

3.3. Initial characteristics of VOO

The initial characteristics of VOO are presented in Table 3. The oxidative and hydrolytic integrity of the oil was confirmed by the low PV, K_{232}, and K_{268} values and by the low contents of free fatty acids which were below the upper legal limit established by IOC for VOO. The VOO contained high amounts of oleic acid (68.21%) and an appropriate ratio of monounsaturated fatty acid (MUFA)-to-PUFA. It also contained considerable amounts of phenolic compounds (286.30±1.16 μg gallic acid equivalents g VOO^{-1}) in the beginning of the assay.

3.4. Determination of oxidative stability of VOO samples

3.4.1. Measurement of PV, AV, and TOTOX values

Primary oxidation products, namely hydroperoxides, were determined by PV measurement. Changes in the PVs of VOO samples during storage at 70 °C are illustrated in Figure 1. The PV of the control increased gradually until the 35th day, indicating the high resistance of VOO to oxidation due to its own (naturally occurring) antioxidants and low unsaturation level. However, the rate of hydroperoxide formation of the control increased sharply after reaching a PV of 38.44 meq O_2·kg^{-1}. This phase (after reaching a PV value of 38.44 meq O_2·kg^{-1}) indicated an accelerated degradation process. VOO supplemented with BHT, *T. vulgaris* EO, and *B. persicum* EO showed lower PVs in comparison with that of the control throughout the storage period. In the initial stages of oxidation, BHT and *T. vulgaris* EOs appear to be slightly more effective than *B. persicum* EO. However, the PVs of the samples containing BHT and *T. vulgaris* EO were significantly lower than that of *B. persicum* EO at the end of storage. This phenomenon may be due to the fact that natural antioxidants in the VOO are consumed at the initial stage of oxidation. Also, this result may be related to the fact that the different magnitudes of antioxidant activities observed among the various antioxidants are more evident at later stages of oxidation.

TABLE 3. Initial characteristics of virgin olive oil (VOO)

Characteristic	Amount	Established limit (IOC, 2015)
Free acidity (%)	1.75±0.22	≤ 2.0
Peroxidevalue (meq O_2 kg^{-1})	4.36±0.27	≤ 20
p-Anisidine value (mg kg^{-1})	3.92±0.08	-
K_{232}	1.49±0.25	≤ 2.6
K_{268}	0.14±0.03	≤ 0.25
Chlorophyll content (mg kg^{-1})	4.73±0.06	-
Carotenoid content (mg kg^{-1})	2.18±0.07	-
Total phenolic content (μg gallic acid equivalents g VOO^{-1})	286.30±1.16	-
Specific gravity	0.913±0.005	-
Refractive index	1.4713±0.0012	-
Palmitic acid C16:0	14.11	7.5–20
Palmitoleic acid C16:1(9)	0.78	0.3–3.5
Stearic acid C18:0	4.07	0.5–5.0
Oleic acid C18:1(9)	68.21	55.0–83.0
Linoleic acid C18:2 (9,12)	11.65	3.5–21.0
α-Linolenic acid C18:3 (9,12,15)	1.19	Max 1.0
Monounsaturated fatty acid/polyunsaturated fatty acid	5.45	

Values given are the means of three replicates ± standard deviation.

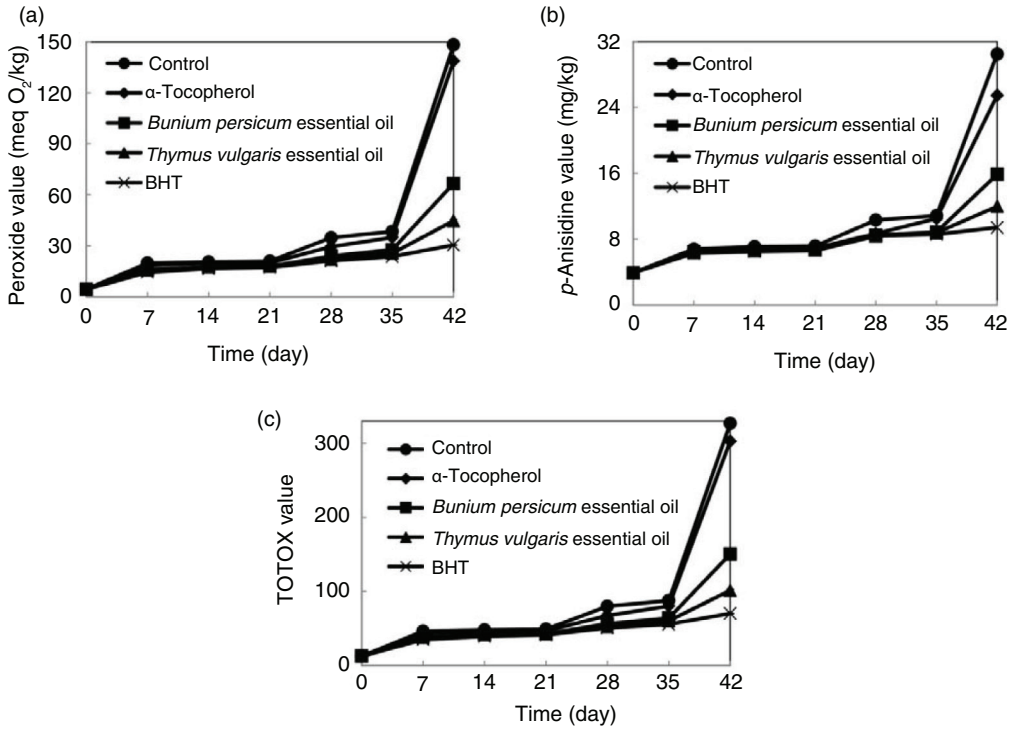

FIGURE 1. Changes in (a) peroxide values, (b) *p*-anisidine values, and (c) TOTOX values of virgin olive oil samples during accelerated storage at 70 °C.

The PV of the sample containing α-tocopherol was slightly lower than that of the control during the entire period of the experiment. The IP_{PV} of the control, BHT, α-tocopherol, *T. vulgaris* EO, and *B. persicum* EO samples were 16.86, 27.08, 18.05, 25.13, and 22.49 days, respectively.

The anisidine test is designed to measure high molecular weights of saturated and unsaturated carbonyl compounds in triacylglycerols (Frankel, 2012). Changes in AVs of VOO samples during storage at 70 °C are illustrated in Figure 1. During the storage, the increasing trend observed for AV was very similar to that obtained for PV.

The control sample exhibited the highest AVs during the entire period of the experiment. Adding *T. vulgaris and B. persicum* EOs offered protection to the VOO, inhibiting the formation of undesirable flavors emanating from secondary lipid oxidation processes. Similar to the PV results, the effects of natural and synthetic antioxidants on delaying the formation of secondary oxidation products was clearly determined at the end of storage, and the order of inhibitory effects of natural and synthetic antioxidants was BHT > *T. vulgaris,* EO > *B. persicum,* EO > α-tocopherol.

The TOTOX value is an indicator of primary and secondary oxidation products. The results of the TOTOX values of the VOO samples during storage at 70 °C are presented in Figure 1. BHT, *T. vulgaris* EO, and *B. persicum* EO all reduced the formation of primary and secondary oxidation products in VOO by 78.51, 69.01, and 53.99%, respectively, when considered at the end of storage. This indicates good capacity of both EOs to inhibit the oxidative process. Thymol and carvacrol are the major components of *T. vulgaris* EO, while γ-terpinene is one of the major components of *B. persicum* EO. These components have been reported to exhibit antioxidant properties (Ruberto and Baratta, 2000). Thymol and carvacrol are primary antioxidants which either delay or prevent the initiation step by reacting with a lipid-free radical or prevent the propagation step by reacting with the peroxy or alkoxy radicals (Yanishlieva *et al*., 1999), thereby retarding VOO oxidation. It has been previously reported that thymol is a better antioxidant in lipids than carvacrol, due to the greater steric hindrance capability of its phenolic group (Yanishlieva *et al*., 1999). The antioxidant activity of γ-terpinene is attributed to the presence of methylene groups in monoterpene hydrocarbons which is strongly active and may compete with the activated methylene in C-11 of linoleic acid (Ruberto and Baratta, 2000).

α-Tocopherol showed no measurable effect in reducing the TOTOX value of VOO. α-tocopherol is reported to possess slight degrees of antioxidant activity and can even be pro-oxidative at times. These results depend on the chemical concentrations and the temperatures applied (Marinova and Yanishlieva, 1992; Schuler, 1990). The threshold value for α-tocopherol as a pro-oxidant in extra VOO oxidation was 60 to 70 ppm during storage at 37 and 75 °C (Deiana *et al*., 2002). Also, it has been reported that the pro-oxidant activity of α-tocopherol tends to decrease as the temperature increases, even at its high levels of concentration (Marinova and Yanishlieva, 1992).

3.4.2. Measurement of K_{232} and K_{268}

The formation of conjugated dienes in fats or oils gives rise to an absorption peak at 232 nm in the ultraviolet region (Frankel, 2012). Changes in K_{232} of the VOO samples during storage at 70 °C are presented in Figure 2. A significant difference ($p<0.05$) in K_{232} was observed between the control and the samples containing *T. vulgaris* EO and *B. persicum* EO. This indicates the significant antioxidant effect of both EOs ($p<0.05$). The K_{232} value of BHT, *T. vulgaris* EO, and *B. persicum* EO exhibited identical increasing trends in the first 28 days of storage. After that, the K_{232} value of the sample which contained *B. persicum* EO increased faster and reached 6.21±0.09 at the end of storage, whereas K_{232} of BHT and *T. vulgaris* EO reached 4.2±0.98 and 5.3±0.07, respectively. The α-tocopherol had no measurable effect on causing a decrease in the formation of the conjugated dienes of VOO during storage at 70 °C.

Changes in K_{268} of VOO samples are due to the formation of conjugated trienes (Figure 2). In line with the K_{232} results, the levels of conjugated trienes at the end of storage were of lowest value in samples containing BHT followed by *T. vulgaris* and *B. persicum* EO, while the highest levels were found in the control and the sample containing α-tocopherol.

The durations of time that were required to reach the upper legal limits of K_{232} (IP_{K232}) and K_{268} (IP_{K268}) during storage at 70 °C are presented in Table 4. As expected, there was a strong correlation between IP_{PV} and IP_{K232} (R^2=0.985; y=0.432x+3.232). It is also understood that a strong correlation exists between IP_{PV} and IP_{K268} (R^2=0.967; y=0.444x+4.277). Moreover, IP_{K232} and IP_{K268} correlated directly with each other (R^2=0.987; y=1.028x-0.589). However, in all samples, IP_{K232} and IP_{K268} values were lower than that of IP_{PV} (with the average percentage of difference being 41.51 and 48.19%, respectively). Also, it was separately observed that K_{232} and K_{268} reached the upper legal limits of 2.6 and 0.25, respectively, almost concurrently. This indicates that the monitoring of VOO oxidation in terms of K_{232} or K_{268} at 70 °C will lead to the maintenance of stability in the VOO with regard to relatively similar quantities.

IP_{PV}, IP_{K232}, and IP_{K268} were increased by the four antioxidants used in this study by approximately the same order as described before (BHT > *T. vulgaris,* EO > *B. persicum* EO > α-tocopherol). However, in all of the VOO samples under study, the IPE_{PV}

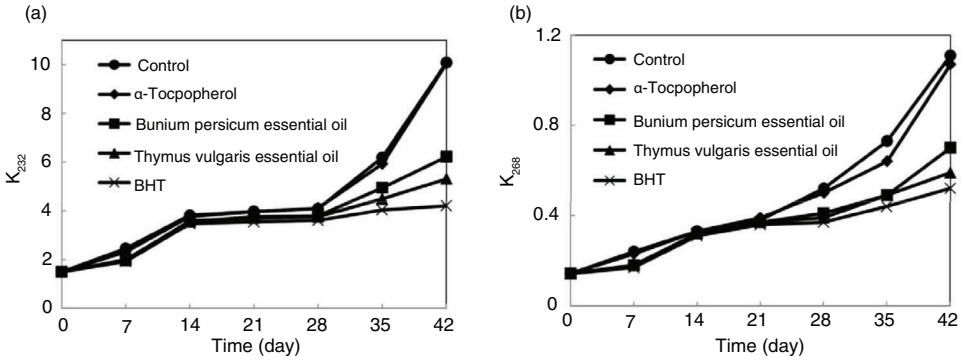

FIGURE 2. Changes in (a) K_{232} and (b) K_{268} of virgin olive oil samples during accelerated storage at 70 °C.

TABLE 4. Duration required to reach the upper legal limits of K_{232} (IP_{K232}) and K_{268} (IP_{K268}) for virgin olive oil samples during storage at 70 °C

			Induction period extension (%)		
Sample	**IP_{K232} (day)**	**IP_{K268} (day)**	**Peroxide value**	**K_{232}**	**K_{268}**
Control	10.54	10.30	0.00	0.00	0.00
BHT	15.16	14.84	60.62	43.83	44.08
Thymus vulgaris essential oil	13.71	13.52	49.05	30.06	31.26
Bunium persicum essential oil	13.13	13.30	33.39	24.57	29.13
α-Tocopherol	11.01	10.54	7.06	4.46	2.28

value was higher than the values for the IPE_{K232} and IPE_{K268} measurements. This indicates that both natural and synthetic antioxidants are more effective in protecting MUFA than PUFA.

3.4.3. Determination of chlorophyll and carotenoid contents

Chlorophyll compounds play an important role in the oxidative stability of VOO due to their antioxidant nature in the dark and their pro-oxidant activity in the presence of light (Criado *et al*., 2008). Carotenoids can act as primary antioxidants by trapping free radicals. They may also act as secondary antioxidants by quenching the singlet oxygen (Liebler, 1993). The chlorophyll contents of VOO samples during accelerated storage are presented in Figure 3. At the end of the storage period, all samples showed a substantial loss in chlorophyll content.

At the end of storage, the chlorophyll contents of VOO samples containing BHT, *T. vulgaris* EO and *B. persicum* EO ultimately decreased by 70.53%, 78.94% and 83.58%, respectively, whereas the chlorophyll content of the sample containing α-tocopherol and the control decreased by 89.68 and 90.91%, respectively. Carotenoid fractions decreased faster than the chlorophyll fraction during oxidation. It is commonly documented that the presence of oxygen and free radicals might accelerate the degradation rate of carotenoids. It is believed that the oxidation of carotenoids depends on the simultaneous oxidation of unsaturated fats (Criado *et al*., 2008). Thus, both oxygen and the presence of free radicals could explain the drastic decrease in carotenoid contents after a short period of storage. BHT, *T. vulgaris* EO, and *B. persicum* EO significantly retarded carotenoid degradation in the treated samples, compared with the control samples and also compared with those containing α-tocopherol. Nonetheless, at the end of storage, there were 28.37, 20.47%, and 13.49% carotenoids remaining in the samples containing BHT, *T. vulgaris* EO, and *B. persicum* EO, respectively, whereas the content of carotenoids was almost completely degraded in the control group and in the sample which contained α- tocopherol.

4. CONCLUSION

According to the results observed in the present research, the inclusion of *T. vulgaris* and *B. persicum* EOs in VOO can retard the lipid oxidation process, thereby delaying the increase in adverse chemical quality parameters (PV, AV, K_{232}, and K_{268}) and protecting the chlorophyll and carotenoid contents of VOO. Also, those EOs rich in phenolic monoterpenes

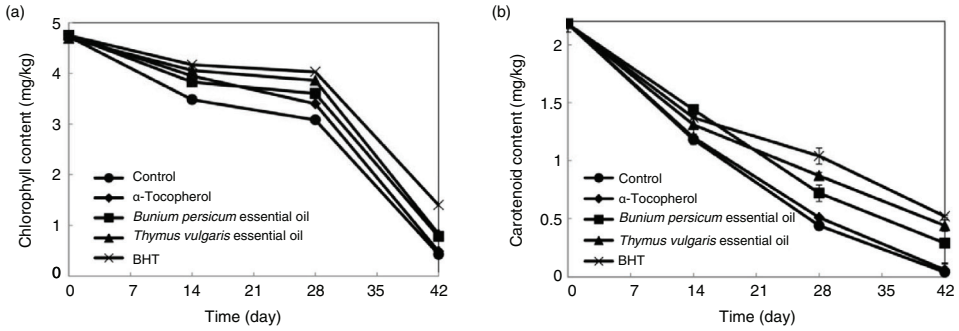

FIGURE 3. Changes in (a) chlorophyll content and (b) carotenoid content of virgin olive oil samples during accelerated storage at 70 °C.

(*T. vulgaris* EO) were found here to be more effective than those rich in monoterpene hydrocarbons (*B. persicum* EO). The effect of *T. vulgaris* EO on retarding the oxidation of VOO in this study was found to be similar to that of BHT. However, BHT is not permitted to be incorporated into VOO. The α-tocopherol had only a small effect on improving the oxidative stability of VOO. Generally, *T. vulgaris* and *B. persicum* EOs can be used as potential natural antioxidants for extending the shelf life of VOO. Further studies at ambient temperatures would be required to determine the actual shelf life of VOOs containing plant EOs.

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