Improving Oxidative Stability of Virgin Olive Oil: Comparison of Zataria Multiflora Essential Oil with α-Tocopherol

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A B S T R A C T

Background and Objectives: Virgin olive oil is a vastly consumed product, with widespread appreciation for its good nutritional and health properties. However, oxidation can reduce its quality. The aim of this study was to investigate how the essential oil of Zataria multiflora (Shirazi thyme) can contribute to the prevention of virgin olive oil oxidation in comparison with the actions of α-tocopherol and BHT. Furthermore, the synergistic activities of citric acid with BHT, Z. multiflora essential oil, and α-tocopherol were investigated.

Materials and Methods: Antioxidant activity of the essential oil was determined using radical scavenging capacity and reducing power assays. Virgin olive oil samples were stored at 60±1 °C in closed amber bottles for 16 days. Oxidation levels of samples were determined by measuring peroxide, anisidine, TOTOX, K232, K266 values, and chlorophyll and carotenoid contents of the samples during the storage period.

Results: Z. multiflora essential oil exhibited a significant radical scavenging capacity and reducing power. Peroxide, anisidine, TOTOX, K232, and K266 values of samples containing Z. multiflora essential oil were significantly lower than those of the control group (without antioxidants). Z. multiflora essential oil reduced the oxidation of virgin olive oil to the same extent as BHT did. Z. multiflora essential oil was more effective than α-tocopherol. The synergistic activities between citric acid and the various compounds, i.e. BHT, Z. multiflora essential oil, and α-tocopherol were 2.42%, 4.74%, 1.28% respectively.

Conclusions: In general, Z. multiflora essential oil can be considered as natural antioxidant for the stabilization of virgin olive oil against oxidation.

Keywords: Accelerated storage, Oxidation, Virgin olive oil, Zataria multiflora

Introduction

Virgin olive oil (VOO) is commonly accepted by consumers as an oil produced without any chemical treatments. It has a high oxidative stability, mainly because of two reasons; firstly, it has a high ratio of monounsaturated-polyunsaturated fatty acids and, secondly, it has significant amounts of polyphenol compounds with strong antioxidant activities (1). However, it contains polyunsaturated fatty acids such as linoleic acid and linolenic acid which reduce VOO oxidative stability. The most important cause of VOO quality degradation is oxidation. Oxidation occurs by enzymatic and chemical reaction pathways (2).

In order to improve the stability of fats and oils, synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tert-butyl hydroquinone (TBHQ) are added into fats and oils (3). However, it has been shown that BHA and BHT are not stable at high temperatures (4,5). Furthermore, TBHQ is not allowed for food application in Canada, Japan, and Europe. In addition, BHA has been removed from the list of generally recognized as safe (GRAS) compounds (6). These health concerns have increased the demand for natural antioxidants (7). Herbs and
spices contain high amounts of biological active compounds with significant antioxidant activity (3).

*Zataria multiflora* belongs to the Lamiaceae family. This aromatic plant is known as Avishan Shirazi. *Z. multiflora* is native to Iran, Pakistan, and Afghanistan (8). The main components of *Z. multiflora* essential oil (EO) are oxygenated monoterpenes thymol and carvacrol, and their corresponding monoterpenic hydrocarbon precursors, i.e. γ-terpinene and *p*-cymene. (9). It has been shown that thymol and carvacrol can pose high antioxidant activities. Thymol and carvacrol can donate hydrogen atoms from their phenol hydroxyl groups to peroxyl radicals and terminate lipid peroxidation chain reactions (10, 11).

Many researches have investigated the effectiveness of different herbs and spices on the stability of vegetable oils. It has been observed that Ajwain EO (0.075%) lowers the oxidation of sunflower oil to a higher extent than BHA and BHT (0.02%) did (12). Oregano EO was effective against extra VOO oxidation (13). Also, there is considerable evidence that shows the synergistic effects of various antioxidants in vegetable oils. Hras et al. reported that rosemary extract showed synergistic effects with ascorbic palmitate and CA in sunflower oil (14). Also, it has been reported that a combined mixture of honey and mint exhibited a synergistic activity and could be used as a promising food additive (15).

The goal of this study was to investigate the effect of *Z. multiflora* EO on VOO oxidation in comparison with *α*-tocopherol and BHT. Also, the synergistic effects of CA with *Z. multiflora* EO, BHT, and *α*-tocopherol were investigated.

**Materials and Methods**

**Materials:** Dried aerial parts of *Z. multiflora* were purchased from a local market in Shiraz, Iran. The genus and species of *Z. multiflora* was authenticated by taxonomists from the Herbarium of Biology Department at Shiraz University, Shiraz, Iran. The VOO was provided by Etka Oil organization. Chemicals such as *α*-tocopherol, 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH°), BHT, TBHQ, potassium ferricyanide, sodium phosphate, trichloroacetic acid, iron (III) chloride, copper (II) chloride, neocuprine (≥98%), ammonium acetate, and Folin–Ciocalteu reagent were purchased from Sigma-Aldrich (St. Louis, MO). Potassium iodide, cyclohexane, methanol, isoctane, hexane, and methanol were purchased from Merck (Darmstadt, Germany).

**Extraction of EO:** *Z. multiflora* EO was extracted according to the method described by Golmakani and Rezaei (9) using a Clevenger-type apparatus. Briefly, 50 grams of *Z. multiflora* sample was mixed with 500 mL of distilled water and hydrodistilled for 3 h. EO samples were dried over anhydrous sodium sulphate and were stored at -18 °C until further analysis (9).

**Gas Chromatography (GC) Analysis of EO:** GC analysis of the EO was carried out using the method described by Khajehie et al. (16). The EO constituents were identified 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). HP-5MS capillary column (5% Phenyl Polysilphenylene-siloxane, 30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness, Agilent Technologies, Santa Clara, CA) was used. The carrier gas was Helium at a flow rate of 0.9 mL/min. In addition, quantitative determination of EO constituents was carried out under the same chromatographic conditions using a GC equipped with a flame ionization detector (FID). For each compound, the relative data for percentage was determined from the electronic integration of the chromatogram’s peak areas.

**Antioxidant Activity of EO**

**Free Radical Scavenging Capacity:** The free radical scavenging capacities of BHT and *Z. multiflora* EO were determined according to the method of Farahmand et al. (17), using the DPPH° assay. The results were expressed as IC_{50} value, which is defined as the amount of sample concentration that can scavenge 50% of DPPH°.

**Reducing Assay:** The ferric reducing antioxidant power (FRAP) of the EO was measured using the procedure described by Farahmand et al. (17), by reducing the Fe (III) to Fe (II). The cupric ion reducing antioxidant capacity (CUPRAC) of the EO was measured by reducing the Cu (II) to Cu (I), using the method of Apak et al. (18). In both FRAP and CUPRAC assays, vitamin C was used as the positive control. FRAP and CUPRAC results were expressed as mg of vitamin C equivalents per gram of sample (mg VCE/g).
Initial Quality of VOO

Free Fatty Acid Content: Free fatty acids were measured according to the AOCS official method (Cd 3d-63). The free fatty acid content was calculated as the percentage of oleic acid (19).

Fatty Acid Analysis: Fatty acid analysis was carried out using the method of Golmakani et al. with some modifications (20). In order to prepare fatty acid methyl esters, VOO was mixed with methanol: acetyl chloride solution at the ratio of 95:5 v/v. The mixture was sealed in a PTFE-lined vial and heated at 85 °C for 1 h. The vial was cooled and 5.0 mL double distilled water was added. Afterwards, 1.0 mL of hexane containing 0.01% TBHQ was added to the mixture. Finally, 1.0 mL of hexane containing 0.01% TBHQ was added again and centrifuged at 4000 xg at 25 °C for 5 min. The upper phase (hexane layer) obtained after centrifugation contained mixtures of fatty acid methyl esters.

To determine fatty acid composition of VOO, a GC (3420A, Beifen system, China) equipped with a FID was used. A BPX70 capillary column (Bis-cyanopropylsiloxane-silphenylene, 30 m × 0.25 mm internal diameter with 0.25 μm film thickness) was used for the separation of fatty acid methyl esters. The column initial temperature was held at 140 °C for 5 min. After that, it was increased to 180 °C at a rate of 20 °C/min. It was held at 180 °C for 9 min. finally, it was increased to 200 °C at a rate of 20 °C/min and held at that temperature for 3 min. The injector and detector temperatures were set at 250 and 300 °C, respectively. One μL of fatty acid methyl ester samples was injected in to the GC system in split mode (at 1:10 ratio). The carrier gas was nitrogen.

Calculation of Iodine Value: Iodine value (IV) was calculated according to the equation described by Kyriakidis and Katsiloulis according to eq. (1).

\[ IV = xC1 + yC2 + zC3; \quad x = 0.95, \quad y = 1.6, \quad z = 2.62. \]  
\[ \text{eq. (1)} \]

C1, C2, and C3 correspond to the sum of mono-, di-, and tri-unsaturated fatty acid methyl esters, respectively (21).

Total Phenolic Content: Total phenolic content of VOO was determined using the Folin–Ciocalteu assay according to the method of Casal et al. (22). Results were expressed as μg of gallic acid per g of VOO (μg GAE/g).

Oxidation Indices of VOO: The peroxide value (PV) was measured using the AOCS official method (Cd 8-53). PV results were expressed as milliequivalents of active oxygen per kg of VOO (19). The anisidine value (AV) was measured using the procedure described by the AOCS official method (Cd 8-53). AV results were expressed as mg per kg of VOO (19). The TOTOX value was calculated using eq. (1) (10).

\[ \text{TOTOX value} = \text{AV} + 2\text{PV} \quad \text{eq. (1)} \]

The K_{232} and K_{268} extinction coefficients were measured following the AOCS official method (ch 5-91) (18). Chlorophyll and carotenoid contents were determined using the procedure described by Minguez-Mosquera et al. (23).

Oxidative Stability of VOO during Accelerated Storage: Z. multiflora EO, α-tocopherol, and BHT were added to the VOO in concentrations of 1000, 100, and 100 ppm, respectively. Also, mixtures of the EO, α-tocopherol, and BHT with 100 ppm CA were added to the VOO. VOO samples were placed into amber bottles (60 mL). The bottles were completely filled and sealed. No headspace was left in the bottles. The samples were kept in an incubator at 60±1 °C for 16 days. The oxidation level of VOO samples was determined by measuring PV, AV, TV, K_{232}, and K_{268} values every 4 days. Also, chlorophyll and carotenoid contents were determined at the beginning and at the end of the storage period.

The IP is defined as the number of days taken for a sample to reach a PV of 20 meq O₂/kg (11).

Protection factor (PF) was calculated using eq. (2).

\[ \text{PF} = \frac{\text{IP}_a}{\text{IP}_v} \quad \text{eq. (2)} \]

where IP, is the induction period (IP) of the VOO sample without antioxidant (control) and IP_a is IP of the VOO samples containing antioxidant (BHT, α-tocopherol, and Z. multiflora EOs) (13). The effectiveness of antioxidants can be determined using the following scale for the PF values: 1.0-1.5 (very low), 1.5-2.0 (low), 2.0-2.5 (medium), 2.5-3.0 (high), and >3.0 (very high) (24).

Antioxidant activity (AA) functions by the concentration of the antioxidant. AA was determined following eq. (3).

\[ \text{AA} = \frac{\text{IP}_a - \text{IP}_v}{[\text{AH}]} \quad \text{eq. (3)} \]

Where [AH] is the antioxidant concentration in ppm unit (25).

Also, the synergistic activity was calculated according to eq. (4).
eq. (4)

\[
\text{Synergism (\%)} = \left( \frac{(IP_2-IP_0)-(IP_1-IP_0)-(IP_2-IP_1)}{IP_2-IP_0} \right) \times 100
\]

Where \( IP_1 \) is the induction period of the sample treated with the mixture of the antioxidant with CA, \( ip_1 \) is the induction period of the sample treated with the antioxidant without CA, and \( IP_2 \) is the induction period of the sample treated with CA (14).

**Statistical Analysis:** All results are reported as the mean value ± standard deviation of triplicate measurements. In order to compare the mean values, a general linear model (GLM) procedure from SAS (Statistical Analysis Software, version 9.1; SAS Institute Inc. Cary, NC) was used. Microsoft Office Excel 2010 was used to calculate the regression equations of the oxidation indices from the storage period, but after 12 days of storage, the PV of the sample treated with CA, and IP \( ip_1 \) during the storage period. The PV of the control increased considerably at the early stages of the storage period, but after 12 days of storage, the PVs of samples containing CA, BHT, and \( \alpha \)-tocopherol were significantly higher than those with CA.

**Results**

**Analysis of EO:** The chemical composition of \( Z. \) *multiflora* EOs is shown in Table 1. \( Z. \) *multiflora* EO contained high amounts of oxygenated monoterpenes and made up 69.49% of the EO. The monoterpane hydrocarbons made up 27.46% of the \( Z. \) *multiflora* EO, while sesquiterpene hydrocarbons comprised 2.97%. The main components of \( Z. \) *multiflora* EO were thymol (52.80%), \( \beta \)-caryophyllene (11.74%), carvacrol (10.33%), and \( \gamma \)-terpinene (6.06%).

**Antioxidant Activity of EO:** \( Z. \) *multiflora* EO was able to scavenge DPPH\(^*\) with \( IC_{50} \) value of 0.06 mg/ml. Comparison of the DPPH\(^*\) scavenging capacity of the investigated EO and those expressed by BHT (\( IC_{50}=0.02 \) mg/ml) showed that the EO possessed slightly lower scavenging activities than BHT. Ferric reducing power and cupric ion reducing power of \( Z. \) *multiflora* EO were 815.96±86.44 and 895.43±10.09 mg of vitamin C equivalents/g sample, respectively.

**Initial Quality of VOO:** PV, AV, Free acidity, \( K_{232} \), and \( K_{368} \) of VOO were 4.45±0.68 meq O\(_2\)/Kg, 3.62±0.12 mg/kg, 1.69±0.22%, 1.67±0.25, and 0.16±0.1, respectively, at the beginning of the storage period. Oleic acid was the major fatty acid (69.72±1.70%) followed by palmitic (18.67±2.68%), linoleic (8.94±2.20%), stearic (1.72±0.14%), and linolenic (0.95±0.63%) acids. The iodine value of VOO was 83.3. Total phenolic content of VOO was 290.5±1.1 (µg GAE/g).

**Oxidative Stability of VOO during Accelerated Storage**

**PV, AV, and TV:** Changes in PVs of VOO samples during storage are illustrated in Figure 1a and Figure 1d, respectively. PVs measured in the control were higher than those samples containing \( Z. \) *multiflora* EO and BHT during the storage period. The PV of the control increased considerably at the early stages of the storage period, but after 12 days of storage, the PV of the control began to decrease. In fact, after 12 days of storage, the formation of hydroperoxides was slower than their decomposition into secondary oxidation products. In samples containing \( Z. \) *multiflora* EO, BHT, and \( \alpha \)-tocopherol, it was observed that the PV reached 12.88±0.54, 12.46±0.06, and 18.10±0.14 meq O\(_2\)/kg, respectively, after 16 days storage. The PVs of samples containing antioxidants without CA were significantly higher than those with CA.

AVs of VOO samples are presented in Figure 1b and Figure 1e. The AV of the control sample increased gradually after 8 days of storage. Sharp increase in the AV of the control sample was observed after 12 days of storage. These findings support the idea that hydroperoxides start to decompose and form secondary oxidation products after 12 days of storage (26). BHT, \( Z. \) *multiflora* EO, and \( \alpha \)-tocopherol significantly inhibited the formation

### Table 1. Chemical composition of \( Z. \) *multiflora* essential oil using gas chromatography

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Retention index</th>
<th>Relative peak area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( \alpha )-Thujene</td>
<td>924</td>
<td>0.31</td>
</tr>
<tr>
<td>2</td>
<td>( \alpha )-Pinene</td>
<td>932</td>
<td>3.35</td>
</tr>
<tr>
<td>3</td>
<td>Camphene</td>
<td>951</td>
<td>0.31</td>
</tr>
<tr>
<td>4</td>
<td>( \beta )-Pinene</td>
<td>975</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>Myrcene</td>
<td>989</td>
<td>2.46</td>
</tr>
<tr>
<td>6</td>
<td>3-Öctanol</td>
<td>993</td>
<td>0.07</td>
</tr>
<tr>
<td>7</td>
<td>( \alpha )-Phellandrene</td>
<td>1004</td>
<td>0.34</td>
</tr>
<tr>
<td>8</td>
<td>( \alpha )-Terpinene</td>
<td>1015</td>
<td>2.21</td>
</tr>
<tr>
<td>9</td>
<td>( \beta )-Cymene</td>
<td>1025</td>
<td>11.74</td>
</tr>
<tr>
<td>10</td>
<td>Limonene</td>
<td>1028</td>
<td>0.63</td>
</tr>
<tr>
<td>11</td>
<td>( \gamma )-Terpinene</td>
<td>1058</td>
<td>6.06</td>
</tr>
<tr>
<td>12</td>
<td>cis-Sabinene hydrate</td>
<td>1065</td>
<td>0.28</td>
</tr>
<tr>
<td>13</td>
<td>Linalool</td>
<td>1098</td>
<td>1.31</td>
</tr>
<tr>
<td>14</td>
<td>Bornol</td>
<td>1164</td>
<td>0.95</td>
</tr>
<tr>
<td>15</td>
<td>Terpinene-4-ol</td>
<td>1175</td>
<td>1.87</td>
</tr>
<tr>
<td>16</td>
<td>( \alpha )-Terpineol</td>
<td>1189</td>
<td>0.88</td>
</tr>
<tr>
<td>17</td>
<td>Thymol</td>
<td>1291</td>
<td>52.8</td>
</tr>
<tr>
<td>18</td>
<td>Carvacrol</td>
<td>1305</td>
<td>10.33</td>
</tr>
<tr>
<td>19</td>
<td>Thymol acetate</td>
<td>1354</td>
<td>0.92</td>
</tr>
<tr>
<td>20</td>
<td>Carvacrol acetate</td>
<td>1371</td>
<td>0.15</td>
</tr>
<tr>
<td>21</td>
<td>(( E ))-Caryophyllene</td>
<td>1417</td>
<td>1.98</td>
</tr>
<tr>
<td>22</td>
<td>Aromadendrene</td>
<td>1436</td>
<td>0.29</td>
</tr>
<tr>
<td>23</td>
<td>( \alpha )-Humulene</td>
<td>1451</td>
<td>0.18</td>
</tr>
<tr>
<td>24</td>
<td>allo-Aromadendrene</td>
<td>1458</td>
<td>0.25</td>
</tr>
<tr>
<td>25</td>
<td>Viridiflorene</td>
<td>1492</td>
<td>0.27</td>
</tr>
</tbody>
</table>
of secondary oxidation products in comparison with the control sample. By the end of the storage period, the corresponding percentage of inhibition by Z. multiflora EO, BHT, and α-tocopherol were 19.87%, 23.99%, and 14.59%, respectively, compared to the control. In the case of samples containing antioxidants with CA, the corresponding percentage of inhibition performed by Z. multiflora EO, BHT, and α-tocopherol were 22.27%, 28.79%, and 16.51%, respectively, compared to the control.

TVs of VOO samples are illustrated in Figure 1c and Figure 1f. TVs of all samples were increased during storage. Z. multiflora EO and BHT were the most stable during the storage period. The effect of Z. multiflora EO was comparable to those of BHT when lowering the TV of VOO. The α-tocopherol was less effective than BHT and Z. multiflora EO.

Antioxidant indices of VOO samples are shown in Table 2. Z. multiflora EO significantly increased the IP of VOO in comparison with that of the control. No significant difference was observed between IP of samples containing BHT with CA and those containing Z. multiflora EO with CA. Therefore, the mixture of Z. multiflora EO and CA can be used to replace synthetic antioxidants, i.e. BHT. The α-tocopherol significantly increased the IPs of VOO in comparison with that of the control, but they were less effective than Z. multiflora EO and BHT.

According to PF results, the BHT and Z. multiflora EO, in addition to the mixtures of these antioxidants with CA, exhibited the highest PF in VOO during storage period. The α-tocopherol exhibited a lower PF than the value exhibited by BHT and Z. multiflora EO.

Figure 1. Effect of Zataria multiflora essential oil, BHT, and α-tocopherol on peroxide values, anisidine values, and TOTOX values of virgin olive oil samples with (d-f) and without (a-c) citric acid.
Table 2. Antioxidant indices and synergism activity of virgin olive oil samples during storage period

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Induction period (day)</th>
<th>Protection factor</th>
<th>Antioxidant activity</th>
<th>Synergism activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without citric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.56±0.17</td>
<td>1.00±0.00</td>
<td>0.00±0.00</td>
<td>-</td>
</tr>
<tr>
<td>BHT</td>
<td>30.84±1.28</td>
<td>4.07±0.08</td>
<td>307.99±7.77</td>
<td>-</td>
</tr>
<tr>
<td>Zataria multiflora essential oil</td>
<td>28.21±0.88</td>
<td>3.73±0.20</td>
<td>27.31±0.33</td>
<td>-</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>16.20±0.14</td>
<td>2.13±0.04</td>
<td>113.22±5.06</td>
<td>-</td>
</tr>
<tr>
<td>With citric acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.12±0.03</td>
<td>1.00±0.00</td>
<td>0.00±0.00</td>
<td>-</td>
</tr>
<tr>
<td>BHT</td>
<td>32.00±0.99</td>
<td>3.93±1.06</td>
<td>146.91±5.23</td>
<td>2.42±0.72</td>
</tr>
<tr>
<td>Zataria multiflora essential oil</td>
<td>29.83±0.63</td>
<td>3.67±0.06</td>
<td>24.29±0.54</td>
<td>4.74±0.69</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>16.81±0.24</td>
<td>2.04±0.01</td>
<td>53.44±1.02</td>
<td>1.28±0.71</td>
</tr>
</tbody>
</table>

*Values are presented as mean ± standard deviation. In each column, means with different letters are significantly different (P<0.05).

Antioxidant activity (AA) of BHT and α-tocopherol was significantly higher than that of Z. multiflora EO. This may be due to the fact that AA depends on the concentration of antioxidant (25). The AAs of samples containing BHT and α-tocopherol without CA were significantly higher than those with CA, but there was no significant difference between the AA of samples containing Z. multiflora EO – samples without CA and those combined with CA.

Synergism activities of tested antioxidants with CA are shown in Table 2. The synergistic activities of CA with Z. multiflora EO, BHT, and α-tocopherol were 4.74, 2.42, and 1.28%, respectively. Thus, when the tested antioxidants were combined with CA, they showed a synergism in VOO.

Figure 2. Effect of Zataria multiflora essential oil, BHT, and α-tocopherol on K232 and K268 values of virgin olive oil samples with (c, d) and without (a, b) citric acid.

K232 and K268: K232 and K268 values of VOO samples during the storage period are shown in Figure 2. Similar to PV results, K232 and K268 values of samples containing Z. multiflora EO was lower than samples of the control and samples containing α-tocopherol. Z. multiflora EO delayed the formation of conjugated dienes and trienes to the same extent as BHT did. The CA showed a low synergistic activity with the tested antioxidants in lowering the K232 and K268. The times required to reach the upper permitted limits of K232 and K268 for VOO samples during the storage period are shown in Table 3. The durations of time that were required to reach the upper permitted limit of K232 and IP correlated directly with samples containing CA (R²= 0.998; y=0.796x+0.160) and also those without CA (R²= 0.989; y= 0.912x+0.251). There was a direct correlation between the durations of time that were required to reach the upper permitted limit of K232 and IP correlated directly with samples containing CA (R²= 0.998; y=0.796x+0.160) and also those without CA (R²= 0.989; y= 0.912x+0.251). In addition, the durations of time that were required to reach the upper permitted limit of K232 and K268 correlated directly with samples containing CA (R²= 0.964; y=3.301x-6.184) and those without CA (R²= 0.991; y=3.670x-6.343).
Thymol showed a high free radical scavenging activity with an IC50 value of 29.77±1.74 mg/mL. According to Yanishlieva et al. (28), thymol, carvacrol, γ-tocopherol, and p-cymene are the main components of thyme EO. Golmakani and Rezaei reported that among the main components of thyme EO, thymol showed a high free radical scavenging activity with an IC50 value of 29.77±1.74 mg/mL. Also, Mishra et al. (29) reported that among the main components of thyme EO, thymol showed a high free radical scavenging activity with an IC50 value of 0.109 μL/mL, whereas p-cymene showed moderate radical scavenging capacity with an IC50 value of 19.6 μL/mL (28). Also, according to Yanishlieva et al., thymol showed a higher radical scavenging activity than carvacrol. This is due to the greater steric hindrance of the phenolic group in thymol compared to carvacrol (29).

**Discussion**

**Analysis of EO:** The main components of Z. multiflora EO were oxygenated monoterprenes (thymol and carvacrol) and monoterpane hydrocarbon (p-cymene and γ-terpinene). Thymol was the predominant compound in the present study, which was also considered as the main component of Z. multiflora EO by Saei-Dehkordi et al. (27). Also, Golmakani and Rezaei reported that Z. multiflora EO is predominantly composed of thymol, carvacrol, γ-terpinene, and p-cymene (9).

**Antioxidant Activity of EO:** Z. multiflora EO exhibited a significant DPPH scavenging activity. The IC50 value of Z. multiflora EO in the present study (0.06 mg/mL) was similar to those reported by Sharififar et al. (30) (0.02 mg/mL). Mishra et al. reported that among the main components of thyme EO, thymol showed a high free radical scavenging activity with an IC50 value of 0.109 μL/mL, whereas p-cymene showed moderate radical scavenging capacity with an IC50 value of 19.6 μL/mL (28). Also, according to Yanishlieva et al., thymol showed a higher radical scavenging activity than carvacrol. This is due to the greater steric hindrance of the phenolic group in thymol compared to carvacrol (29).
**Z. multiflora** EO showed a significant ability to donate electrons to free radicals, converting them into more stable non-reactive species and terminating the free radical chain reactions. It has been reported that **Z. multiflora** EO showed a significant reducing power. **Z. multiflora** EO showed a higher reducing power than *Bunium persicum* EO. The reducing powers of both EOs were lower than TBJHQ (31).

**Initial Quality of VOO:** At the beginning of experiment, VOO met the criteria set by the International Olive Council (IOC) for the virgin category regarding the value of free acidity (≤ 2.0) (32). Also, PV, K$_{232}$, and K$_{268}$ were below the upper permitted limit established by IOC for VOO (PV≤20 meq O$_2$/kg, K$_{232}$≤2.6, and K$_{268}$≤0.25). Therefore, the initial oxidation level of VOO was low. VOO contains a high ratio of monounsaturated/polyunsaturated fatty acids and a low iodine value. This fact is accompanied by a significant amount of total phenols with potential antioxidant capacities, and it results in a high oxidative stability of the VOO. However, presence of polyunsaturated fatty acids can reduce oxidative stability of VOO.

**Oxidative Stability of VOO during Accelerated Storage**

**PV, AV, and TV:** Adding the **Z. multiflora** EO and BHT to the samples significantly decreased the PV of samples in comparison with the control during storage. After 16 days storage, the PV did not exceed the upper limit (20 meq O$_2$/kg) permitted by IOC for VOO (32). This indicates that **Z. multiflora** EO is effective in preventing the formation of hydroperoxides. Similarly, Simandi et al. reported that 0.6 % thyme extract (*T. vulgaris*) reduced the sunflower oil oxidation to the same extent as 0.1 % BHT did (33). **Z. multiflora** EO was more effective than α-tocopherol in lowering the PV of VOO. Samotyja and Malecka reported that α-tocopherol was less effective in sunflower oil stabilization in comparison with rosemary and blackcurrant seed extracts (34).

**Z. multiflora** EO, BHT, and α-tocopherol were effective against formation of secondary oxidation products, mainly in aldehydic structure. Asensio et al. showed that extra virgin olive oil samples added with oregano EO had lower anisidine values than the control during storage (13). Also, Vahidyan et al. showed that the effect of **Z. multiflora** EO (500 ppm) in reducing the formation of secondary oxidation products in mayonnaise formulated with linseed oil was similar to that of BHA (1000 ppm) (35).

**Z. multiflora** EO significantly inhibited the TV of VOO in comparison with the control sample. The antioxidant capacity of an EO depends on its chemical composition. Thymol and carvacrol can react with lipid-free radicals and delay the initiation step of oxidation. Also, they can react with peroxy or alkoxy radicals and prevent the propagation step of oxidation. The inhibitory effect of γ-terpinene against lipid oxidation is due to the presence of a strongly activated methylene group which can compete with the activated methylene in the C-11 of linoleic acid, and inhibit linoleic acid oxidation (36).

Combinations of antioxidants with CA showed a synergistic effect on lowering the TV of VOO. Similarly, Hras et al. reported that the synergistic activity of rosemary extract combined with CA was 2.61% in the sunflower oil (14).

The effectiveness of different antioxidants commonly corresponds to increasing the IP. This delay is often expressed as PF (14). **Z. multiflora** EO and BHT and their combination with CA showed a "very high" antioxidant activity (PF>3). Similarly, Bandonien et al. reported that sage, sweet grass and tansy extracts exhibited very high (PF>3) antioxidant activity (25). α-Tocopherol both with and without CA showed "medium" antioxidant activity (PF of 2.0-2.5).

Since **Z. multiflora** EO reduced the VOO oxidation at higher concentrations, **Z. multiflora** showed lower AA than that of BHT. It is commonly known that the maximum permitted levels of synthetic antioxidants are established based on various toxicological parameters. However, these parameters and limitations do not need to be applied to naturally occurring compounds. Therefore, **Z. multiflora** EO can be used at higher levels, compared with those of synthetic counterparts, thereby increasing their antioxidant effectiveness.

**K$_{232}$** and **K$_{268}$:** During the unsaturated fatty acids oxidation, the rearrangement of the double bonds, produce conjugated dienes and conjugated trienes. Conjugated dienes in fats and oils absorb at 232 nm. Similarly, conjugated trienes absorb at 268 nm (26). **Z. multiflora** EO, BHT, and α-tocopherol were effective in lowering K$_{232}$ and K$_{268}$ of VOO. Ayadi et al. reported that extra VOOs treated with thyme,
rosemary and basil exhibited a significant low value of $K_{232}$ and $K_{270}$ than that of the control during the storage period (37). In this study, the time needed to reach the upper permitted limits of $K_{268}$ was shorter than those of $K_{232}$ and PV. Absorbance at 268 nm is due to the oxidation of linoleinic acid in VOO (26). Similarly, Gomez-Alonso et al. showed that after 21-month storage of VOO at room temperature, linoleinic acid reduced 2.5–2.8 times greater than linoleic acid (38). The time required to reach the upper permitted limit of $K_{232}$ in samples treated with $\alpha$-tocopherol were significantly shorter than the durations required by BHT and *Z. multiflora* EO. However, durations of time that were required to reach the upper legal limit of $K_{268}$ in samples treated with $\alpha$-tocopherol were slightly shorter than the durations required by BHT and *Z. multiflora* EO. This observation shows that the activities of different antioxidants differ depending on the oxidizing substrate.

**Chlorophyll and carotenoid contents:** *Z. multiflora* EO can be proposed as natural additives for the preservation of VOO color quality. Also, Asensio et al. reported that chlorophyll and carotenoid contents of extra virgin olive oil samples containing oregano EO was higher than that of control during storage (13).

In this study, effect of *Z. multiflora* EO was investigated on VOO oxidation in comparison with the effects of $\alpha$-tocopherol and BHT. Also, the synergistic activity of CA was investigated on natural and synthetic antioxidants. Results showed that *Z. multiflora* EO significantly reduced VOO oxidation. This can be attributed to the presence of several antioxidant compounds in *Z. multiflora* EO. The *Z. multiflora* EO reduced VOO oxidation to the same extent as BHT did. The $\alpha$-tocopherol was less effective than *Z. multiflora* EO. Also, CA exhibited a synergistic activity with natural and synthetic antioxidants. Generally, *Z. multiflora* EO can be used as natural antioxidants to reduce VOO oxidation and to preserve the color of VOO. Further research will be required to determine the correlation between the antioxidant activity and the chemical composition of *Z. multiflora* EO.

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