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## Original Article

# Eugenol and Clove (*Syzygium aromaticum*) Essential Oil Efficacy on Oxidative Stability of Sunflower Oil during Accelerated Storage

**Fereshteh Ahmadian[1], Majid Aminzare[1], Mehran Mohseni[2], Mirjamal Hoseini[3] and Hassan Hassanzadazar[1*]**

*[1]Department of Food Safety and Hygiene, School of Public Health, Zanjan University of Medical Sciences, Zanjan, Iran*
*[2]Department of Food and Drug control, School of Pharmacy, Zanjan University of Medical Science, Zanjan, Iran*
*[3]Department of Pharmacology and Toxicology, School of Pharmacy, Zanjan University of Medical Sciences, Zanjan, Iran*

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***Corresponding author**
Hassanzadazar_h@zums.ac.ir

**ABSTRACT**

This study aimed to formulate the Sunflower Oil (SFO) by Eugenol (EUG) as a substitute for clove essential oil (CEO) to extend the stability of SFO during accelerated storage. The oxidation rate in supplemented samples of SFO with 50, 150 and 300 µg/ml concentrations of the CEO and EUG was evaluated by measuring Peroxide, Anisidine and Totox values during the 12 days with three-day intervals under accelerated storage. Induction time for primary oxidation of the samples was measured by rancimat apparatus. Eugenol (75.29%), Caryophyllene (11.81%), Eugenol acetate (6.98%) and α-Humulenen (2.94%) were the major constituents of CEO, respectively. Peroxide, Anisidine and Totox values increased in all treatments and had an upward trend during storage from zero to 12th days. In comparison, EUG containing treatments at 150 µg/ml had the lowest amount of Peroxide, Anisidine and Totox values with significant difference than CEO containing treatments at the last day of storage period ($P<0.05$). The treatments containing 150 µg/ml concentrations of CEO and EUG showed the highest stability.

## INTRODUCTION

Vegetable oils and fats are the main sources to provide essential fatty acids for human body [1]. The sunflower oil (SFO) as a widely used oil in food industry and cooking is one of the appreciated sources of the higher content of polyunsaturated fatty acids such as linoleic acid. The presence of unsaturated fatty acids in this type of oils makes them susceptible to lipid peroxidation. Lipid peroxidation is an important factor in reducing the nutritional quality of food systems, including edible oils and fats [1]. It has detrimental effects on the human health due to the formation of toxic products which causes aging, cardiovascular diseases, joint inflammation, brain deterioration, cell membrane and DNA damage that lead to the carcinogenesis, mutagenesis and many other problems [2].

Various synthetic antioxidants used widely as food additives to stabilize, increasing shelf life and improving the quality of edible oils and fats in the food industry, including tertiary butyl hydroquinone (TBHQ), butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and propyl gallate (PG) [3]. Due to the enhanced knowledge and demand of food consumers', several researchers focus on finding natural antioxidants as new sources to improve harmful risks of synthetic antioxidants in human health [4-6]. Plant origin, extracted compounds are known as the main sources of natural antioxidants containing phytochemicals and phenolic compounds that can reduce lipid peroxidation and could be used as a potential alternative for the synthetic antioxidants [4]. In general, an increasing attention is now paid to the antioxidant properties of essential oils (EOs) and herbal extracts to increase sensory attributes and the acceptability of customers. Several items can influence the natural antioxidants' efficacy in the prevention of lipid oxidation in edible oils: The type and structure of antioxidant, the oil constituents including fatty acid profile and the components such as tocopherols [7].

Clove or *S. aromaticum* (L.) Merr. & L.M.Perry (*Eugenia caryophyllata*) is aromatic dried flower buds of the Myrtaceae family and native to tropical regions [8]. Clove extracted compounds demonstrate remarkable antioxidant effects. The clove essential oil (CEO) has been classified and recognized as a natural generally recognized as safe (GRAS), an authorized and safe food additive by the Food and Drug Administration (FDA) [9]. Several components identified in clove extracts and EO including hydrolysable tannins, phenolic acids and flavonoids. Eugenol is reported as the major component of this herb [8]. In addition to its use as a flavoring in food, there are several reports on pharmacological and biological activities of clove extracts and EO and Eugenol such as antioxidant, antidiabetic, anti-inflammatory, anticancer, hepatoprotective, antinociceptive, hypolipidemic effects and antiviral, antifungal, antibacterial, anticarminative, antispasmodic, analgesic, antiseptic, and insecticidal effects [9-12].

Preservative effects of clove extracts and essential oil were demonstrated in meat [13], lettuce leaves [14], soybean oil [15] and Eugenol in mayonnaise [8]. Although the antioxidant activity of the CEO has been reported in some studies, but there is no report about the suitable concentrations of the CEO and EUG as the major compound of clove oil added into SFO at accelerated storage. On the other hand, the aim of this study was to formulate SFO by EUG as a replacement of CEO to extend the stability of SFO during accelerated storage and determine the most suitable concentration of CEO and EUG added into SFO compared to TBHQ as a synthetic antioxidant used in the oil industry.

## MATERIALS AND METHODS

### Materials

Purified SFO without antioxidants was obtained from the koorosh Food Industry Company (koorosh, Qazvin, Iran). CEO (*S. aromaticum* EO) was obtained by hydrodistillation of the dried buds of clove tree purchased from the local markets in Zanjan. All other used chemicals were provided from the Sigma Company (Sigma–Aldrich GmbH, Sternheim, Germany) and the synthetic TBHQ was purchased from Yasho industries (Yasho, Mumbai, INDIA).

Identification of the EO composition

The components of purchased EO were determined using Gas Chromatography/Mass Spectrometry (GC/MS) analysis. A Hewlett Packard 5890 gas chromatography equipped with a GC Column HP-5MS (Length 30 m× ID 0.25 mm ×Film thickness 0.25 µm) was used to analyze EO composition, according to the described method by Aminzare *et al*. 2017[16]. Helium flow rate as carrier gas was 1 ml/min. Initially, column temperature was set to 50 ◦C and gradually was enhanced to 120 ◦C at a 2 ◦C/min rate, retained for 2 min, and increased to 300 ◦C. Ionization energy of 70 eV mentioned to e MS procedure and compound identification were conducted by comparing their retention indices with authenticated samples and mass spectral data existing in the Wiley library (Wiley Registry 11th Edition software, Weinheim, Germany).

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The best concentrations of CEO and EUG to add in SFO and evaluating oxidative stability during accelerated storage were determined using the DPPH method. The radical scavenging activity of different concentrations of CEO and Eugenol (200, 150, 100 and 50 µg/ml for Both CEO and EUG) was measured. DPPH• scavenging activity was conducted according to the described method by Golchin *et al*., 2012 [17]. In this method, electron or hydrogen atoms donating of bioactive materials was investigated based on the discoloration of a methanolic reagent of DPPH (purple). Briefly, 50 µL EO was added to 5 ml of a methanolic solution of DPPH (0.004 %). This mixture was shacked and incubated in a dark place at room temperature for 30 minutes. Then optical absorbance of the solutions was read at 517 nm with a spectrophotometer (Pharmacia LKB, Novaspec II, Sweden). Methanol was used to zero the spectrophotometer. BHT was used as standard antioxidant. DPPH radical inhibition activity was calculated based on the following formula:

Inhibition rate (%) = (A *Blank*–A *Sample*/A *Blank*) ×100

### Preparation of Ooil Samples

Clove EO and EUG were added to preheated refined SFO in the dark brown bottles at 50, 150 and 300 µg/ml concentrations chosen based on DPPH radical scavenging assay. The total volume of each sample was 200 ml. TBHQ was used as a synthetic antioxidant at a concentration of 100 µg/ml (The recommended concentration for edible oil industry

based on Iran national standard No. 3608). All samples were stored and subjected to accelerated oxidation in an oven at 65°C for 12 days. Immediately, oil samples were tested at 3-day intervals. According to the previous reported researches, one day of storage in this temperature is equal to one month of storage at ambient temperature [18]. The oxidation in supplemented samples of SFO was evaluated by measuring peroxide, Anisidine and Totox indices. Induction time for primary oxidation of the samples was measured by rancimat apparatus.

### Analytical Procedures

Peroxide value (PV) and Anisidine value (AV) were measured according to AOCS official methods NO. cd 8-53, 1998 and cd 18-90, 1990, respectively [19]. Totox value (TV) was also calculated based on the described method by Shahidi and Wanasundara (2002) [20]. The Totox Index is a measure to show total oxidation, including primary and secondary products of oxidation, which is calculated by following formula [21]: Totox = 2 (PV) + AV

Oxidative stability index (OSI) of lipid in supplemented SFO was ascertained using a rancimat apparatus (Metrohm Model 892, Herisau, Switzerland) in accordance described method by Farahmandfar *et al*. 2015 [22]. The rancimat method as an accelerated aging test is a technique can predict the oxidative stability of oils and measures the secondary metabolites produced from the oxidation of oils and fats, including aldehydes, ketones, and acids. Three grams of each oil sample were placed into rancimat vessels to measure the thermal resistance of the supplemented oils. Oxidation resonant conditions such as high temperature (110 °C) and air flow at a specific time are used (25 L/Sec). In this method, the air flow is guided by the oxidation reaction and releasing volatile compounds to the solute containing distilled water resulted enhancement of the water electrical conductivity which were plotted against time (h). The time (hour) it takes from the moment of onset to the sudden rise in electrical conductivity is called the induction time or period of induction (The oxidative stability of oil).

### Statistical Analysis

The results were reported as mean ± SD (standard deviation). One-way Anova analysis was used to compare mean values by SPSS software (Ver. 18, Chicago, USA). Tukey test at a 95% confidence level was also used to determine the significant differences between the means. All experiments were performed in three replicates.

## RESULTS AND DISCUSSION

### Chemical Ccomposition of CEO

Four main compounds containing 97.02 % of the total EO were determined using GC-MS method. Eugenol (75.29 %), Caryophyllene (11.81 %), Eugenol acetate (6.98 %) and α-Humulenen (2.94 %) were major constituents of the EO, respectively (Table 1). The retention time and Kovats index of these compounds were 34.06, 36.47, 38.01, 40.66 minutes and 1376, 1502, 1524 and 1562, respectively.

This result is consistent with the reported results of Razafimamonjison *et al*. 2014, Guan *et al*. 2007, Chaieb *et al*. 2007; Prashar *et al*. 2006; Pawar and Thaker, 2006 and Lee and Shibamoto, 2001 (Table 2) [10, 23-27]. As is shown in the table 1, the proportion of compounds has been reported differently in various studies that can be due to the differences in plant varieties, geographical location, environment, climatic and seasonal conditions, soil type and growth stages of the plant [28,29].

### DPPH Radical Scavenging Ability

In this method, DPPH radicals react with antioxidants to a reduced form, and their color turns from dark purple to light yellow, resulting in a decrease in absorption at 515-517 nm wavelengths. Therefore, the higher absorbance rate indicates the remained content of DPPH and the lower ability of the antioxidants in the removing of free radicals. The inhibition power of the clove EO and EUG against DPPH free radicals has been demonstrated in table 2. CEO and EUG had the highest effect at 150 µg/ml concentration.

As is shown in table 3, DPPH radical scavenging effect of CEO and EUG were constant at the concentrations higher than 150 and 100 µg/ml, respectively. It was the main reason to choose a lower (50 µg/ml) and a higher concentration (300 µg/ml) than 150 µg/ml to prepare treatments in this study. Gulcin *et al*. 2012 showed that CEO has an inhibition effect on the DPPH radicals, superoxide anion radicals and hydrogen peroxide [17]. Ozjan and Arslan, 2011 reported antioxidant effect of CEO at lower concentrations in the hazelnut and poppy

oils which is inconsistent with our result that can be due to the CEO composition [15]. Eugenol (4-alkyl-2-methoxyphenol) is the main composition of CEO. Phenols can reduce oxidative deterioration by their antioxidant and free radical scavenging abilities [29]. This ability is commonly due to their redox-oxidation characteristics, which are mediated by several mechanisms such as the free radical scavenging activity, metals chelating activity and single oxygen extinguishing capacity [13, 30, 31]. These numerous antioxidant activities are also comparable with the other antioxidant compounds such as alpha-tocopherol, BHA and BHT.

## Peroxide Value (PV)

The changes of PV in the studied samples are shown in Figure 1. Peroxides and hydroperoxides are known as primary products of lipid oxidation, which broke down at the end stages of oxidation [32]. Obtained results showed that the PV increased in all treatments and had an upward trend during storage from the zero to 12th days. This raising occurred more rapidly in the samples without antioxidant (control). At first day, the peroxide content of the control sample did not differ significantly with the other treatments. At the third day onwards, it showed a significant difference ($P < 0.05$) (Figure 1). Peroxide value was 7.67–12.2 meq/kg in the bioactive containing samples (stabilized samples) after 9 days of storage, which increased to 15.4 at 12th day. The maximum PV was 18.64 meq/kg for control sample at the last day of storage period.

**Table 1** Chemical composition of clove essential oils

| NO. | Components | % | Retention time (RT) | Kovats Index (KI) |
|---|---|---|---|---|
| 1 | Pyridine | 0.51 | 5.12 | 720 |
| 2 | Nonanal | 0.49 | 20.65 | 1093 |
| 3 | Decanal | 0.12 | 26.30 | 1207 |
| 4 | 2*E*-Dodecenal | 0.11 | 29.23 | 1269 |
| 5 | Undecanal | 0.09 | 31.31 | 1314 |
| 6 | Eugenol | 76.07 | 34.12 | 1377 |
| 7 | Caryophyllene | 8.72 | 36.47 | 1502 |
| 8 | a-Humulenen | 4.95 | 38.02 | 1524 |
| 9 | Aceteugenol | 5.17 | 40.66 | 1562 |
| 10 | Pentadecanal | 0.16 | 47.98 | 1726 |
| - | Total | 96.39 | - | - |

**Table 2** Comparison of main compounds of clove essential oil reported in different studies

| Main components | References |
|---|---|
| Eugenol (75.29%), Caryophyllene (11.81%), Eugenolacetate (6.98%), α-Humulenen (2.94%) | Present study |
| Eugenol (88.58%), Eugenyl acetate (5.62%), β - Caryophyllene (1.39%), 2-Heptanone (0.93%), Ethyl hexanoate (0.66%), Humulenol (0.27%), α-Humulene (0.19%), | [9] |
| Eugenol (77.50-79.87%), Eugenyl acetate (9.56-16.01%), β-caryophyllene (4.06-6.91%) | [21] |
| Eugenol (48.82- 58.2), Caryophyllene (13.99-36.94), Eugenol acetate (3.89- 22.34) | [22] |
| Eugenol (78%), β-Caryophyllene (13%) | [23] |
| Eugenol (47.64%), Benzyl alcohol (34.10%) | [24] |
| Eugenol (69.8%), β-Caryophyllene (7.3%), α-Humulene (0.8%), Acetyl eugenol (20.9%) | [25] |

**Table 3** DPPH radical scavenging ability of clove essential oil and Eugenol (%)

| Bioactive ingredient | Concentration (mg/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 10 | 20 | 30 | 40 | 50 | 100 | 150 | 200 |
| Clove EO | 19.2±0.09aa | 39.9±0.06ba | 57.2±0.1ca | 73.1±0.2da | 83.6±0.24ea | 93.7±0.1fa | 95.1±0.02ga | 95.1±0.1ga |
| Eugenol | 28.8±0.1ab | 43.2±0.1bb | 63.4±0.08cb | 84.1±0.13db | 93.7±0.2eb | 96.1±0.23fb | 96.1±0.05ga | 96.1±0.01ga |
| TBHQ | - | - | - | - | 91.6±0.2ac | 95.6±0.15bb | - | - |

Capital letters in each raw show significant difference between treatments of each bioactive material

Small letters in each column show significant difference between bioactive materials

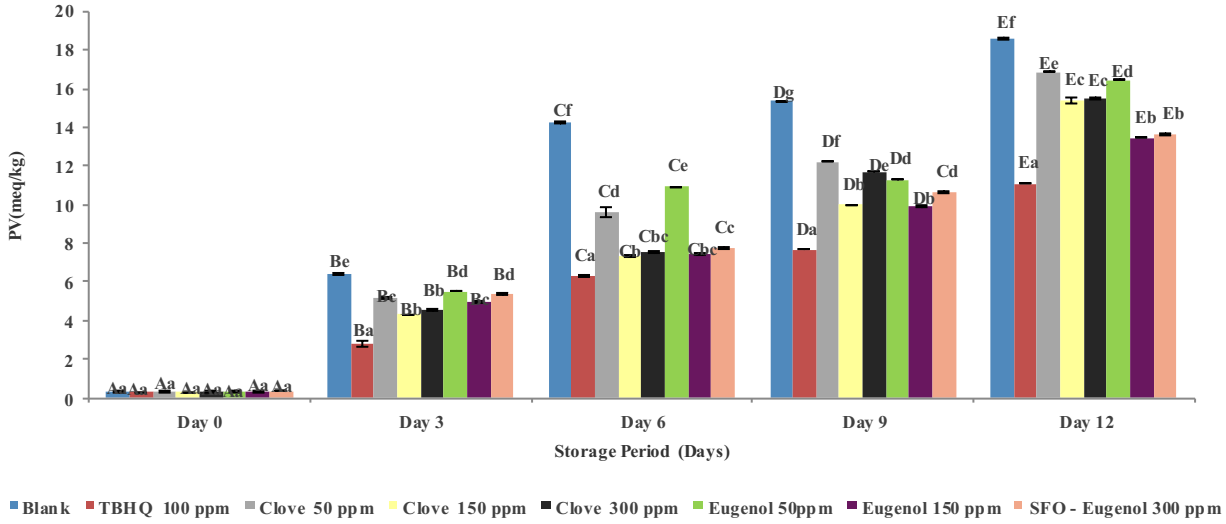


**Fig. 1** Changes in peroxide value (PV) of control and Sunflower oil treatments during accelerated storage

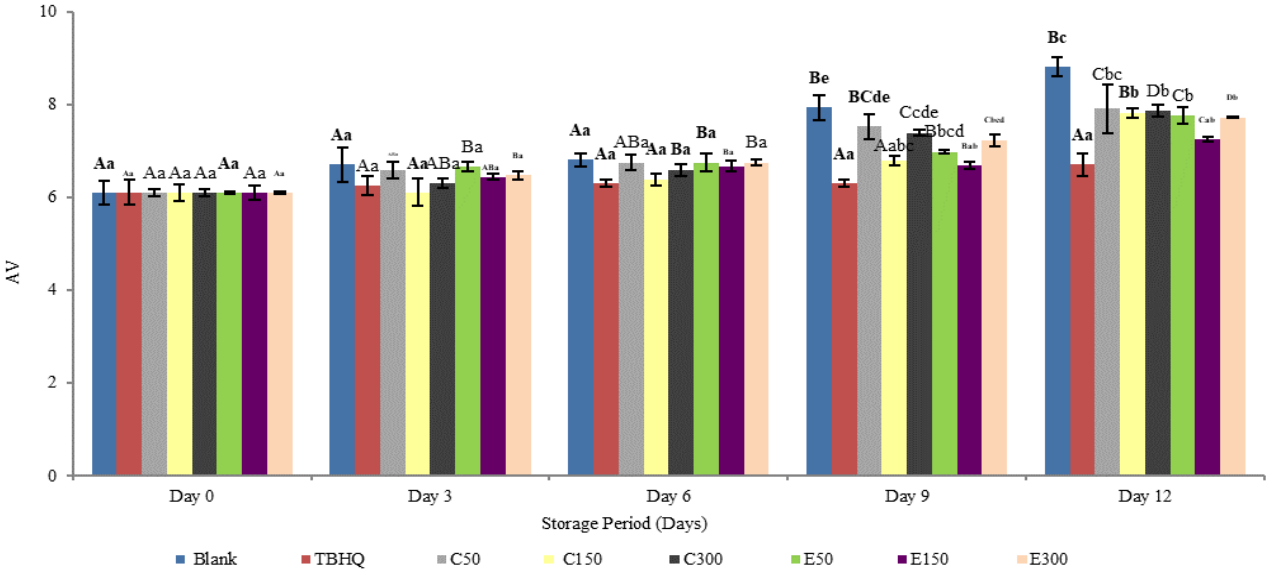


**Fig. 2** Changes in p-Anisidine value (P-AV) of control and Sunflower oil treatments during accelerated storage
**C50**: Clove essential oil 50 µg/mL; **C150**: Clove essential oil 150 µg/mL; **C300**: Clove essential oil 300 µg/mL; **E50**: Eugenol 50 µg/mL; **E150**: Eugenol 150 µg/mL; **E300**: Eugenol 300 µg/mL

All concentrations of CEO and EUG reduced the oxidation rate of SFO during the storage period under accelerated conditions. The SFO samples containing CEO at 150 µg/ml showed the lowest PV. The same regular results were observed for EUG containing samples. Until the 6th day, the lowest PV belonged to the CEO at concentration 150 µg/ml, However, on the 9th and 12th days of storage, the sample containing 150 µg/ml of EUG showed lower peroxide levels than the other treatments. In addition to EUG, other phenolic compounds such as beta-Cariophyllene and alpha-Homulenene can control PV in the samples after the 6th day of storage. It should be considered that the phenolic compounds are active during a specific period as antioxidants, and, over time, their impress declines and eventually becomes ineffective. In other word, such antioxidants inhibit oil spoilage in the early stages, thereby delaying the onset of the oxidation but they were decomposed over time [3, 32]. Some compounds exhibit pro-oxidative properties including phenolic compounds at high concentrations [33]. The increase in PV in treatments containing 300 ppm of CEO and EUG in the 12th day of storage can be attributed to this reason.

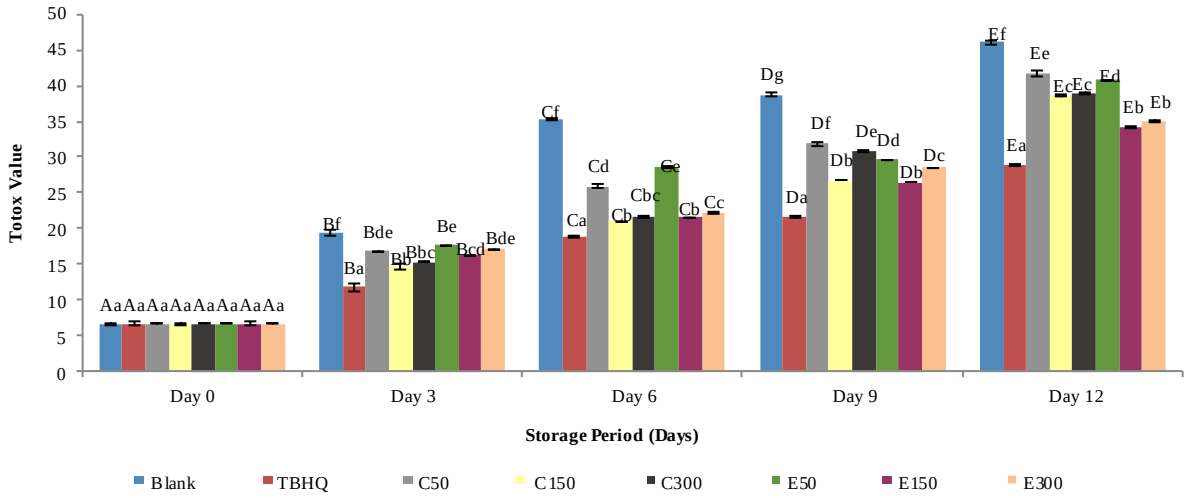


**Fig. 3** Changes in Totox value (TV) in control and Sunflower oil treatments during accelerated storage
**C50**: Clove essential oil 50 µg/mL; **C150**: Clove essential oil 150 µg/mL; **C300**: Clove essential oil 300 µg/mL; **E50**: Eugenol 50 µg/mL; **E150**: Eugenol 150 µg/mL; **E300**: Eugenol 300 µg/mL

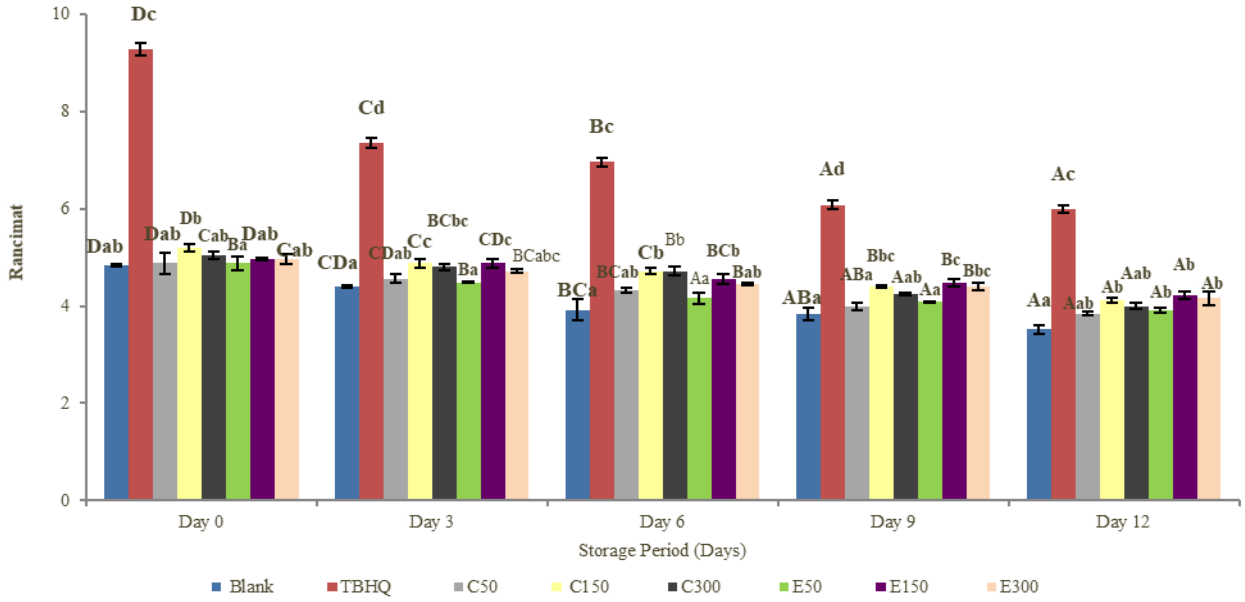


**Fig. 4** Changes in oxidative stability in control and Sunflower oil treatments during accelerated storage
**C50**: Clove essential oil 50 µg/mL; **C150**: Clove essential oil 150 µg/mL; **C300**: Clove essential oil 300 µg/mL; **E50**: Eugenol 50 µg/mL; **E150**: Eugenol 150 µg/mL; **E300**: Eugenol 300 µg/mL

These results are in agreement with the obtained results of conducted studies on sunflower and soybean oils by EO of the *ferulago angulata* boiss [1,32].

Relative increase in PV of the control sample was significantly higher than treated SFO samples with the CEO and EUG ($P<0.05$). Although, no studies have been found in our literature review about the stabilization of SFO by the CEO and EUG, but this trend is consistent with the results of the studies conducted to stabilize SFO by different concentrations of *Mentha piperita* and *Ferulago angulate* EOs, respectively [32,34].

Generally, hydroperoxides decompose into secondary oxidation compounds during storage time, but in this study, by the end of storage time, the rate of formation of the hydroperoxides is higher than their decomposition into secondary oxidation products that can be due to the short storage time and low temperature used for storage. All concentrations of CEO and EUG showed less antioxidant effect than TBHQ. This result is in accordance with the reported result of Hashemi *et al*. 2011 conducted to evaluate oxidative stability of SFO supplemented with *Zataria multiflora* Boiss [21].

### P-anisidine Value (AV)

Anisidine analysis is a method to evaluate aldehyde content (2 alkenals, 2 and 4 alkanes) produced by the decomposition of hydroperoxides. This method is more sensitive to unsaturated the aldehydes and is a criterion to investigate the secondary oxidation and evaluation of the resulting products. The oil oxidation continues during storage resulting increase of AV which reaching to maximum at the end of storage time [35].

The results of this study showed that the AV increased in all treatments during the storage period (Figure 2). Changes of the AV were similar in all treatments in days 0, 3 and 6 and no significant difference was observed in the results. ($p> 0.05$) These results can be attributed to the low temperature and short storage period. These conditions are consistent with the rising trend of peroxides content. Their break down and converting to aldehyde and ketone compounds is low. A significant increase in AV was seen in 9th and 12th days in the control samples. The highest and lowest AV were seen in control and TBHQ treatments in both days. Eugenol containing treatments at 150 µg/ml had the lowest amount of AV with no significant difference in comparison with TBHQ containing treatments ($p> 0/05$) (Figure 2).

The duration, temperature, presence of free fatty acids and other peroxidants are the factors influence the oxidation intensity and increase the p-AV in oils [36]. Oxidation of SFO continues and increases with elongation of storage period and as a result, AV increases in all treatments and reaches to maximum at the end of storage period. AV reduced in all treatments of the CEO and EUG which was due to the presence of antioxidants that cause the low oxidation rate and the formation of non-volatile aldehydes. A Similar result was reported by Sadeghi *et al*. 2017 conducted to enhance oxidative stability of SFO by *Ferulago angulate* [32].

### Total Oxidation Value (Totox Value)

Totox value (TV) is a measure of total oxidation includes primary and secondary oxidation products. It is a combination of anisidine and peroxide values [21]. TV is a useful indicator of oil oxidation and its lower value indicates the higher oil's resistance to oxidation [20]. In this study, TV was the same in all treatments on the day 0 of storage period ($p> 0.05$) (Figure 3). As shown in Figure 3, the TV increased with the storage elongation in all studied treatments. TV in the control treatments was significantly ($P <0.05$) higher than the TBHQ treatments from the third day until the end of the storage period (12th day). In comparison, treatments containing 150 µg/ml CEO and 150 µg/ml EUG showed the best effect on different days in accordance with the results of the changes in PV and AV. The results showed that the samples containing synthetic antioxidant (TBHQ) have had the lowest TV since the third day until the end of the storage period ($p< 0.05$). These results are consistent with the reported results of studies conducted to stabilize SFO against oxidation by oil supplementation with different concentrations of *Garcinia mangostana* Linn. peel and *Pleurotus porrigens* extracts [3, 37].

Oxidative Stability Index (OSI)

The oxidative stability of the treated samples is shown in Figure 4. The lowest and highest stability were seen in the control and TBHQ treatments, respectively during accelerated storage. TBHQ containing treatment had significant differences with all treatments ($p< 0.05$). In comparison, treatments containing 150 µg/ml concentrations of CEO and EUG showed the highest stability. According to obtained results, the oxidative stability of oils did not increase after addition of various antioxidants at higher concentrations of bioactive compounds, which may be due to the reduced antioxidant effect and increasing pro-oxidant properties of the bioactive compounds at higher concentrations [32]. It can be concluded that each antioxidant has the greatest effect at a particular concentration. Although the OSI is used for quality evaluation of oils and fats, but at higher temperatures, it is not suitable to determine antioxidant activity. Natural antioxidants are volatile compounds and may be removed from the oil by air flow in this method [30]. The obtained results are consistent with reported results by Sadeghi *et al*. at (2016) and (2017) conducted on Soybean and sunflower oils, respectively [1,31].

## CONCLUSION

In accordance to our observations, it is possible to improve the oxidative stability of SFO by addition CEO and EUG at concentration150 µg/ml which was more effective than other concentrations. Relatively, EUG showed good antioxidative efficacy in comparison with the CEO and TBHQ and its addition to SFO provided acceptable quality of the samples. Investigation of the synergistic effect of a mixture of bioactive compounds (CEO and EUG) in lower concentrations as well as with other natural antioxidants is suggested to stabilize edible oils and to compare and replacement with synthetic antioxidants.

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