Zataria multiflora and *Bunium persicum* Essential Oils as Two Natural Antioxidants

Zangiabadi M (M.Sc.)[1], Sahari MA (Ph.D.)[1]*, Barzegar M (Ph.D.)[1], Naghdi Badi H (Ph.D.)[2]

1- Department of Food Science and Technology, Tarbiat Modares University, Tehran, Iran
2- Department of Cultivation and Development, Institute of Medicinal Plants, ACECR, Karaj, Iran
* Corresponding author: Department of Food Science and Technology, Tarbiat Modares University, Tehran, Iran, Tel: +98-21-48292328; Fax: +98-21- 48292200
Email: sahari@modares.ac.ir

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Abstract

Background: Autoxidation is considered to be the main route of edible oil deterioration, which produces undesirable odors and flavors (attributed to primary and secondary products of unsaturated fatty acids) during storage and heating.

Objective: Antioxidant activities of *Zataria multiflora* (ZMEO) and *Bunium persicum* (BPEO) essential oils (EOs) in linseed oil (containing higher ω-3 in vegetable sources) were studied with 4 indexes.

Methods: Total phenol content, electropositive potential (by bleaching using violet ethanol solution of DPPH˚), antioxidant capacity (by radical cation of ABTS), scavenging activity of hydrogen peroxide and Fe-reducing power of the EOs were measured. Then ZMEO and BPEO (0.6, 1, 1.4, 1.8 mg/ml) were added to linseed oils; peroxide and tiobarbituric acid in 0th- 15th days (at 60 ˚C) were compared with TBHQ (0.01, 0.02) and BHT (0.1, 0.2).

Results: IC_{50} of ZMEO and BPEO in mentioned assays were 0.78 and 1.52; 0.11 and 7.50; 0.31 and 0.72 mg/ml and $RP_{0.5}$ for reducing power assay was 0.76 and 2.12 mg/ml. Total phenolic compound of ZMEO and BPEO were 322 and 50.6 mg/ml. The best antioxidant levels of reductive oxidation were 600 for ZMEO and BPEO and 20 for TBHQ.

Conclusion: PV and TBA values demonstrated that both of EOs in linseed oil had the same antioxidant activity in 600 ppm (ZMEO>BPEO) in 4 tests, and EOs will be used as natural antioxidants as a good replacement in 600 ppm with BHT (100) and TBHQ (10) as synthetic.

Keywords: Linseed oil, *Zataria multiflora*, *Bunium persicum*, Antioxidant activity

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Introduction

Oxidative changes of the fatty foods are considered as the major factor in producing undesired odor and taste and reducing their nutrition value [1]. On other hand, peroxidation of fats doesn't result only in reduction of the quality of foods, but is also seen as leading factor for diseases such as cancer, mutation, aging, and arteriosclerosis [2]. Active oxygen and free radicals are widely believed as disease-bearing and tissue injury factors. Active oxygen, in forms such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\circ-}$), and singlet oxygen ($^1O_2$) produced from natural metabolism, attacks biological molecules and results in cell or tissue injury [2]. In general, antioxidants are defined as compounds which prevent or delay the peroxidation of the fats.

Synthetic antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG), and tertbutylhydroquinone (TBHQ), are used extensively in food industries. However, there are a lot of questions and suspicions about their safety. The researches has shown that BHA has the potential to express the cancer in animals, and it also has shown that BHT in high concentration can cause bleeding externally and internally and finally death in some species of rat and pig due to its effect on blood coagulation factor [1]. Considering the increasing tendency of consumers to use the additives natural resources and increasing awareness of disadvantages of synthetic additives, utilizing essential oils as additives in foods, beverages, and cosmetics are increasing. Regarding the studies done on the essential oils in terms of odor, flavor they give to foods and beverages, they have the potential of being used with several objects. Many researches have addressed to antimicrobial, antifungal, antioxidative, and radical scavenging properties [3].

The medicinal plant of *Zataria multiflora* is cultivated in Iran. The essential oil of *Zataria multiflora* is extracted from the flowered browses of the plant, having the compounds with important pharmaceutical, antimicrobial, and antioxidant effects [4, 5].

The plant of *Bunium persicum* is considered as the herbal, growing as wild plant in dry lands in Iran. The seed of this plant has oily essence, both its seed and essence has pharmaceutical effects [6].

Various tests have measured the antioxidant capacity of tested samples. The tests used in this study included: azinobis (3-ethyl-benzothiazolin-6-sulfonic acid) ($ABTS^{\circ+}$), 2, 2'-diphenyl-1-picrylhydrazyl ($DPPH^{\circ}$), hydrogen peroxide scavenging, and reducing power. Also the total phenolic contents of the essential oils, was determined by Folin-Ciocalteu.

The purpose of this study, in addition to complete the previous studies, to determine the antioxidant capacity of essential oil of *Zataria multiflora* and *Bunium persicum* by $ABTS^{\circ+}$, and $DPPH^{\circ}$, hydrogen peroxide scavenging and reducing power. In addition, to determine the antioxidant capacity of these essential oils in linseed oil (containing higher ω-3 fatty acid in vegetable sources [7]) by peroxide and thiobarbitoric acid values.

Material and Methods

Materials

Materials were purchased from various companies as follows: Chloroform, acetic acid, chloridric acid, potassium iodate, starch,

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sodium thiosulfate, hexane, TBA, BHT, TBHQ, potassium persulfate, ethanol, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, potassium ferricyanide, trichloroacetic acid, ferric choloride, ascorbic acid, carbonate sodium, H_2O_2, Folin-Ciocalteu (FC) reagent from German Company Merk; α-tocopherol and azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (-$ABTS^{\bullet+}$) from Company Fluka; cyclohexane from Company Riedel-dehaen; potassium iodide from German Company Applie Chem.; 2,2-Diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and galic acid from Company Sigma. To prepare the linseed oil (*Linum usitatissimum*), the seed were purchased from market and the oil was extracted in laboratory, that the seeds were cleaned and ground and poured in some cartouches, then some cotton soaked in hexane was put upon them, the extraction was done in the room temperature. The essential oil of *Zataria multiflora* and *Bunium persicum* was purchased from Iranian Company Plant-Essence Gorgan.

Determination of total phenolic content

The total phenol content was measured by Waterhouse (2002) method using reagent of FC as follows [8]:

A 1.54 ml of water was added to 20 µl of sample, and then a 100 µl of FC reagent was added to the solution, then the solution was stirred for 1-8 min at room temperature, after it was added by 300 µl of sodium carbonate and the resulted solution was stirred and maintained at room temperature for 2 hours, and finally its absorption was read at 765 nm. It was prepared gallic acid by concentrations of 50-500 mg/l and applied as standard and the results were reported as mg gallic acid per milliliter sample.

Determining the antioxidant capacity

$DPPH^{\circ}$ test

The electropositive potential of the essential oils was measured by method of bleaching using violet ethanol solution of $DPPH^{\circ}$. The method was done according to Shahsavari *et al.* (2008) with some changes in the method as follows [6]:

A preparation of 2 ml in ethanol was made from each various concentrations, for *Zataria multiflora* (0.1 to 3.0 mg/ml) and for *Bunium persicum* (0.2 to 4.0 mg/ml), and then it was added to 1 ml of 0.2 molar solution of $DPPH^{\circ}$, and finally it was measured the reductive absorption at 517 nm after 1 hour. Ethanol was used as blank and it was measured as control the absorption of ethanol solution of $DPPH^{\circ}$ with no antioxidant.

Inhibition percentage was calculated by the following formula:

$$I\% = 100 \times (A_{blank} - A_{sample}) / A_{blank}$$

Where A_{blank} and A_{sample} are control absorption (the content of all material but the testing compound) and sample absorption, respectively.

$ABTS^{\circ+}$ free radical decolorization assay

Antioxidant capacity was measured by radical cation of ABTS according to Cai *et al.* 2004 [9]. In order to produce the radical cation of ABTS, a 7 mM $ABTS^{\circ+}$ solution was prepared and then added by 2.45 mM of potassium persulfate and left for 12-16 at room temperature, two reagents react stoichiometrically at a ratio of 1:0.5. After that, subscript this solution was diluted by ethanol until absorbance of 0.7 ± 0.05 absorption at 734 nm. Finally, 0.1 ml of sample was added to 3.9 ml of the $ABTS^{\circ+}$solution, and the absorption was read

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after 15 min. All data are mean of three determinations and IC_{50} was calculated for each of them. In addition, vitamin C (µg/ml) was applied as reference standard.

Hydrogen peroxide scavenging activity

The scavenging activity of hydrogen peroxide was determined for two essential oils according to the Büyükbalci and Nehir (2008) with some moditification [10]:

One ml of 0.1- 0.6 and 0.4 - 1.2 mg/ml of ZMEO and BPEO were mixed with 2.4 ml of 0.1 M phosphate buffer (pH= 7.4). Then, 0.6 ml of 43 mM hydrogen peroxide in the same buffer was added and the absorption was read at 230 nm, after 40 min.

Fe-Reducing power

Fe-reducing power of the essential oils (EOs) was determined according to the method of Hsu *et al.* (2006) with some modifications [11]. One ml of various concentrations of sample was mixed with 500 µl of potassium ferricyanide (1% w/w in water) and 500 µl of 0.2 M phosphate buffer, and left for 20 min in water bath at 50 °C. After cooling the solution, it was added 500 µl of trichloroacetic acid (10% w/w) and centrifuged for 10 min. at 3000 rpm. After that, 500 µl of transparent supernatant was separated and then added to 100 µl of ferric chloride and finally, the absorption of solution was read after 30 min at 700 nm. The increase in absorption of reaction mixture indicates the increase in reducing power of essential oils.

Effect of ZMEO and BPEO on oxidative stability of linseed oil

Different amount of ZMEO and BPEO at four levels (0.6, 1, 1.4, and 1.8 mg/ml), TBHQ at two levels (0.01, and 0.02 mg/ml) and BHT at two levels (0.1, and 0.2 mg/ml) were added to linseed oils. After that peroxide value and tiobarbituric acid index were measured in 0th, 3th, 6th, 9th, 12th and 15th days (at 60 °C) according to the methods of AOCS and Madson *et al.* (1998) [12, 13]. All data are mean of triplicate reiteration of the test by three times.

Statistical analysis

The data was analyzed statistically by the SPSS software ver 16. It was used the fully randomized statistical design, and the difference among means were compared using Duncan test at $p<0.05$.

Results

Standard curve equation was given as $r^2=0.9993$, $Y=0.0012X+0.0254$. The results showed that the total phenolic content in ZMEO and BPEO were 322.0± 2.9 and 50.7± 104 mg gallic acid/ml, respectively.

Comparison of DPPH radical scavenging capacity (IC_{50} value) of ZMEO and BPEO with some natural and synthetic antioxidant was presented in Table 1. The $ABTS^{\circ+}$scavenging activity of ZMEO vs. and BPEO vs. was showed in Figures 1 and 2. The Table 2 shows IC_{50} of both studied EOs, compared with TBHQ, α-tocopherol, and vitamin C as reference standard. Figure 3 shows the effect of the EOs concentration (200 and 400 ppm) and TBHQ upon the rate of scavenging activity of hydrogen peroxide. According to this result, BPEO doesn´t show any scavenging activity in concentration of 200 ppm. Table 3 shows IC_{50} for both studied EOs and TBHQ. Table 4 shows the results of reducing power assay ($RP_{0.5AU}$) of ZMEO,

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TBEO and Vit C and Figures 4 and 5 indicate the effect of increasing the concentration of essential oils upon the increasing rate of absorption.

Figures 6 - 9 show the changes of PV and TBA values of linseed oil containing ZMEO and BPEO as two natural antioxidants and synthetic antioxidants after heating at 60 ˚C for 15 days.

Table 1 - Comparison of DPPH radical scavenging capacity (IC_{50} value) of ZMEO and BPEO with some natural and synthetic antioxidants

Sample	IC_{50}	Reference
Zataria multiflora	0.78 ± 0.03^{c} mg/ml	Present study
Bunium persicum	1.52 ± 0.05^{d} mg/ml	Present study
TBHQ	0.002 ± 0.00^{a} mg/ml	Present study
α-tocopherol	0.006 ± 0.00^{b} mg/ml	Present study
The other researches		
Zataria multiflora	22.4 ± 1.0 µg/ml	[21]
Bunium persicum	0.88 ± 0.04 mg/ml	[7]
Origanum	751.5 µg/ml	[16]
Cinnamon bark	90.63 µg/ml	
Ocimum basilicum	47057.45 µg/ml	[22]
Ocimum canum	8343.19 µg/ml	
Red kaprao	343.56 µg/ml	
White kaprao	768.82 µg/ml	

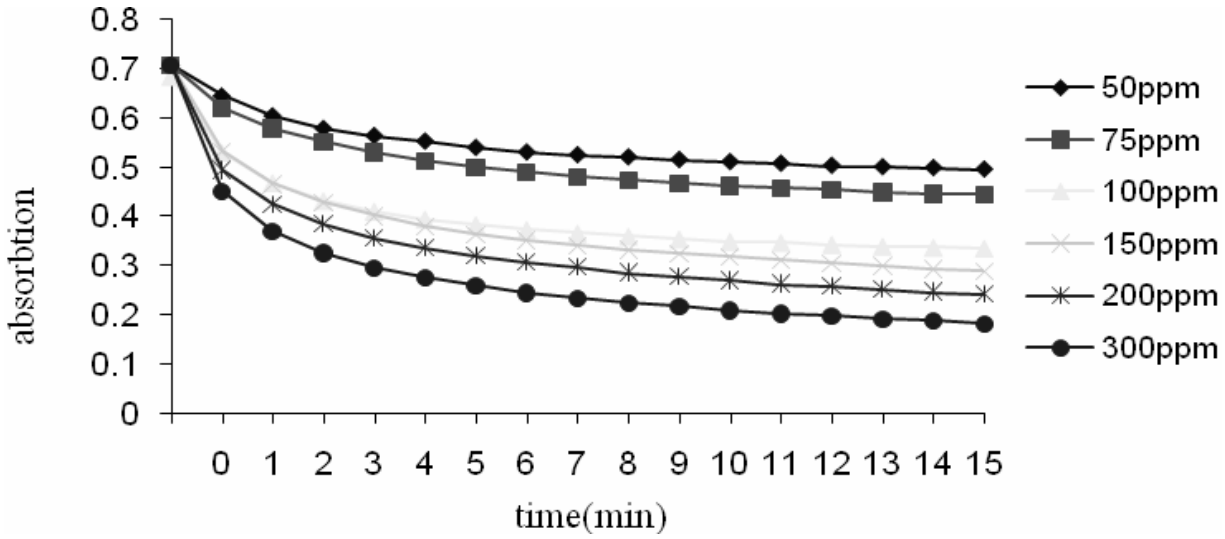

Figure 1- ABTS$^{\circ+}$ scavenging activity of ZMEO vs. time plots

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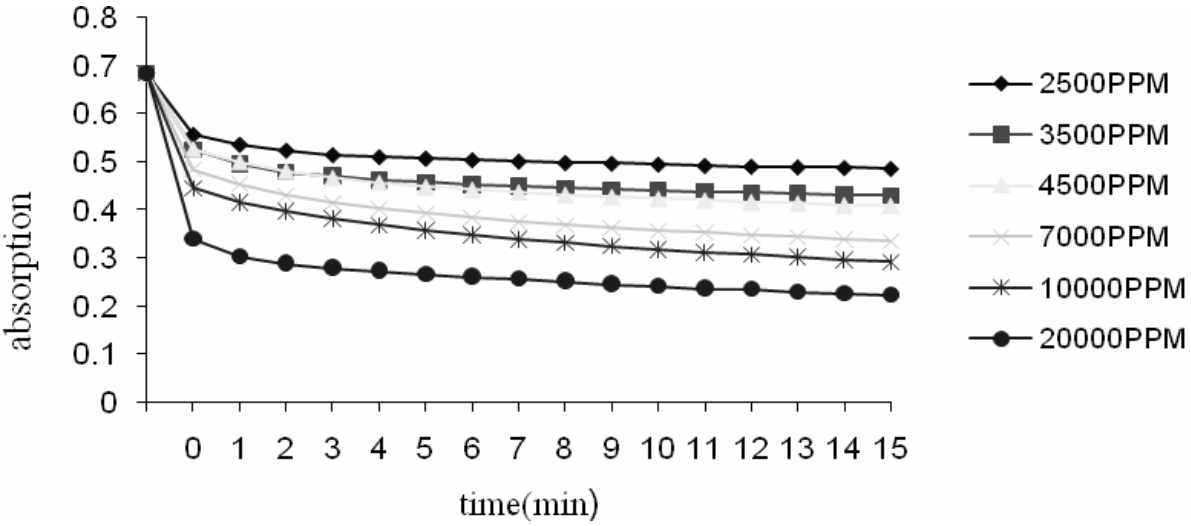

Figure 2- ABTS$^{\circ+}$ scavenging activity of BPEO vs. time plots

Table 2- IC_{50} of both studied EOs and other antioxidants

Sample	IC_{50} (mg/ml)
ZMEO	0.11 ± 0.00^{c}
BPEO	7.5 ± 0.14^{d}
TBHQ	0.04 ± 0.00^{a}
α-tocopherol	0.07 ± 0.01^{b}
Vit C	0.05 ± 0.00

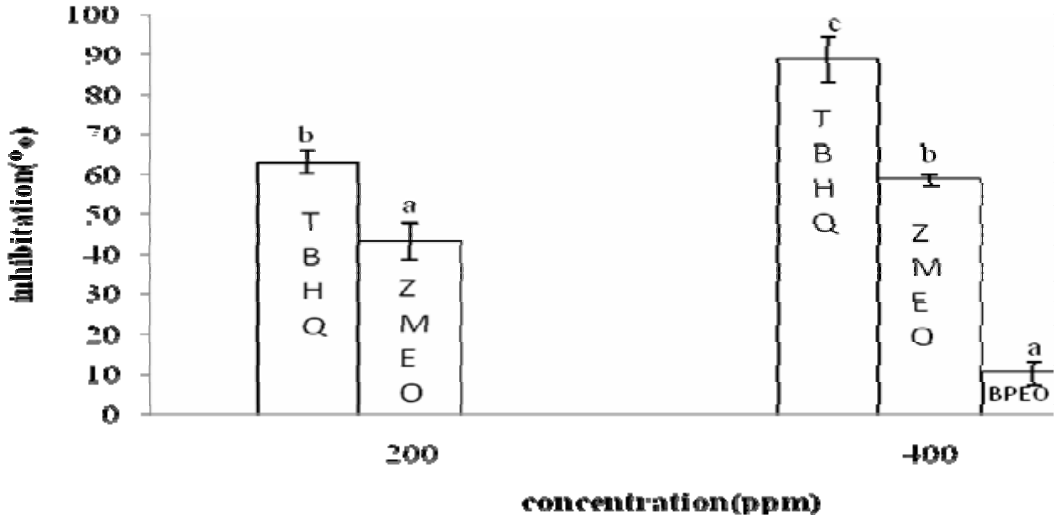

Figure 3- Antioxidant activity of ZMEO and BPEO at different concentrations determined by hydrogen peroxide scavenging method

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Table 3- IC_{50} of both studied EOs and TBHQ in hydrogen peroxide scavenging method

Sample	IC_{50} (ppm)	Reference
ZMEO	312.6 ± 10.8[b]	Present study
BPEO	716.1 ± 21.4[c]	Present study
TBHQ	130.9 ± 6.0[a]	Present study
The other researches		
Extract of *Gmelina arborea*	73.6 ± 0.03	[28]
Extract of *Lagerstroemia reginae*	2121.73 ± 1.5	[29]
Extract of *Excoecaria agallocha*	134.29	[30]
Mentha longifolia essential oil	476.3	[31]
Mentha viridis essential oil	195.1	

Table 4- $RP_{0.5AU}$ values of ZMEO, *BPEO*, TBHQ and Vit C

Sample	$RP_{0.5AU}$ (ppm)	Reference
ZMEO	757.7±27.9[c]	Present study
BPEO	2120.0±9.8[d]	Present study
TBHQ	23.2±1.1[b]	Present study
Vit C	12.6±0.3[a]	Present study
The other researches		
Extract of Doum fruit	978	[11]
Extract of *Garcinia Mangostana*	84.35	[32]
Extract of *Syzygium cuminii*	5.6	[33]

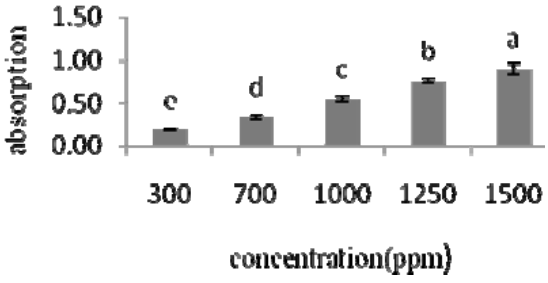

Figure 4- Antioxidant activity of ZMEO in Fe-reducing power assay

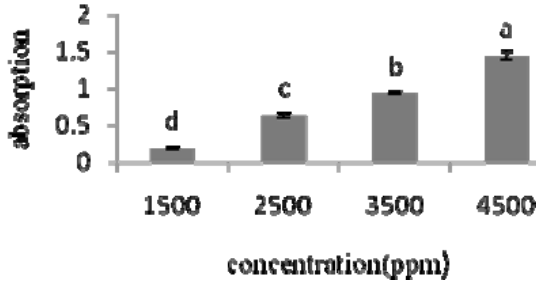

Figure 5- Antioxidant activity of BPEO in Fe-reducing power assay

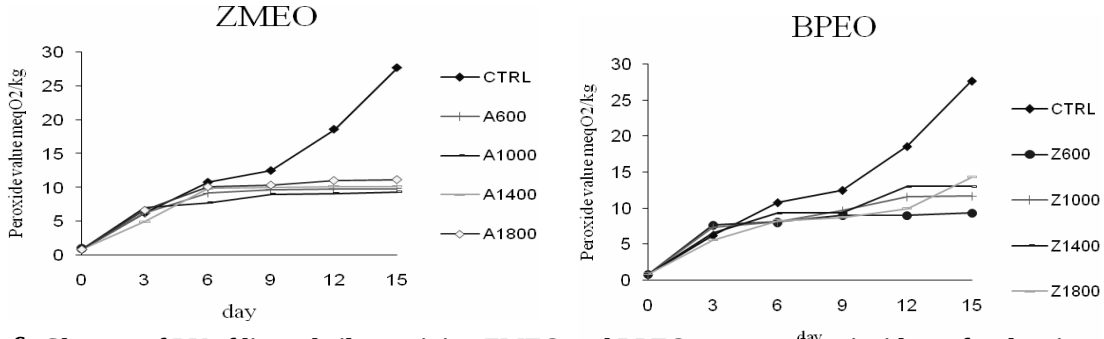

Figure 6- Changes of PV of linseed oil containing ZMEO and BPEO as natural antioxidant after heating at 60˚C for 15 days

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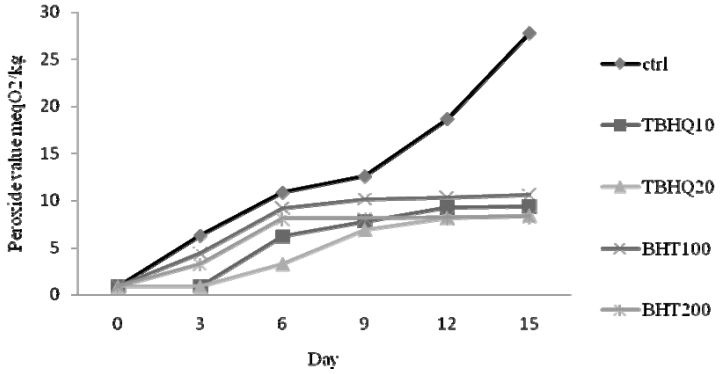

Figure 7 - Changes of PV of linseed oil containing synthetic antioxidants after heating at 60˚C for 15 days

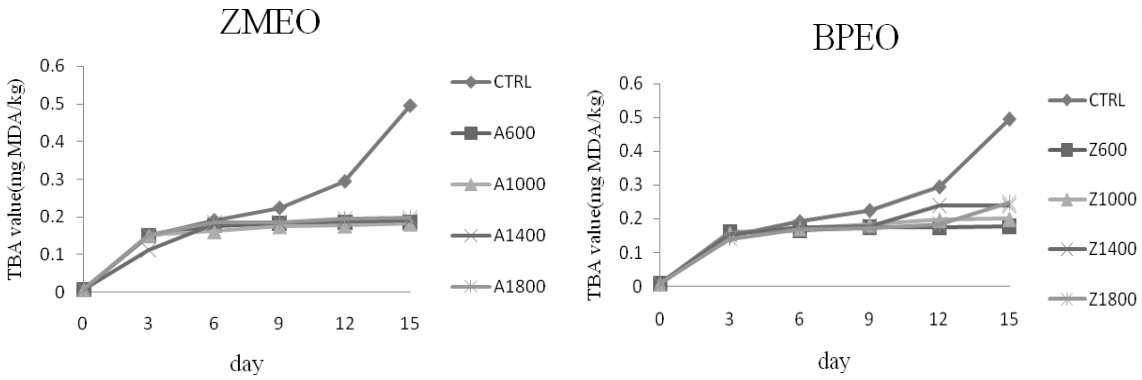

Figure 8 - Changes of TBA value of linseed oil containing ZMEO and BPEO as natural antioxidant after heating at 60˚C for 15 days

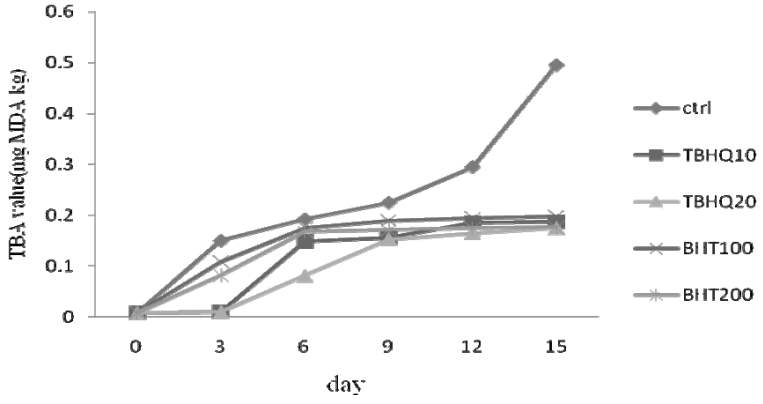

Figure 9- Changes of TBA of linseed oil containing synthetic antioxidants after heating at 60˚C for 15 days

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Discussion

Total phenolic content

The total phenolic content of essential oils was determined by using spectrophotometric method and the reagent Folin-Ciocalteu and gallic acid was used as standard. In a study by Cao *et al*., (2009), the phenol content for essential oil of *Mosla chienesis* was determined as 105.1 µg gallic acid/mg [14]. In 2004, Wangenstein *et al.* showed that the phenol content for coriander was 0.14 g/100g gallic acid [15]. Lin *et al.* (2009) showed in a study on 42 oily essences that the highest phenolic content is seen for origanum, cinnamon bark, and thyme wild by 1107.2, 658.4, 275.5 µg/ml, respectively [16].

The phenol contents are of the important vegetable compounds, because their hydroxyl groups have the inhibitory potential for radicals [17]. Various researches have reported that there is a relation between the phenol content and antioxidant activity, but some researchers showed in their studies that, there may be no relation at all [18].

Hydrogen - donating activity

The inhibitory activity for DPPH˚ is used as general for assessing the antioxidant potential in inhibiting the free radicals. The free radical DPPH˚ is a useful method in assessing the effect of antioxidants, because the DPPH˚ is more stable than hydroxyl and superoxide radicals [19]. To inhibit the radicals is very important in eliminating the harmful effects of existing radicals in foods and biological systems. In DPPH˚ test, antioxidants can reduce the stable radical of DPPH to yellowish diphenilpicrylhydrazine. The test is based on reduction of alcoholic solution of DPPH˚ at presence of antioxidant with hydrogen donating and formation of DPPH-H. The molecule has the absorption at 517 nm in radical state, and turns into a diamagnetic stable molecule after receiving electron or radical of hydrogen [20].

As it is obvious from Table 1, ZMEO and *BPEO* have relative good antioxidant activities and the inhibitory activity of these two essential oils for DPPH radical, even though lower than TBHQ and α-tocopherol, is more than *Ocimum basilicum* and *Ocimum canum*, and ZMEO has the activity similar to white kaparo and origanum [7, 16, 21, 22].

Measurement of radical cation ABTS scavenging activity

Reactivity of $ABTS^{\circ+}$ is more than $DPPH^{o}$ but its mechanism is different. Also, it has more solubility in water. The $ABTS^{\circ+}$ can be prepared using various antioxidants, but the results showed that using $K_2S_2O_8$ as an oxidant increases the rate of $ABTS^{\circ+}$ [23]. This test is applied for determination of antioxidants activity (AOA) that soluble in water and fat [24].

As it is obvious, the following Figures (1 and 2), by increasing in the EOs concentrations results more intensive increasing in absorption reduction. In addition ZMEO shows better antioxidant activity at low concentration than BPEO. On the other hand, gradient of electron giving during the test in ZMEO was smoother than *BPEO*.

The results (Table 2) showed that both studied EOs have lower antioxidant activity than TBHQ and α-tocopherol. ZMEO has more antioxidant activity than BPEO. Considering that free radicals are major factor in peroxidation of fats in chain reactions, inhibiting the free radicals directly can enhance the quality of nutrition materials;

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therefore it is common to make use free radicals of DPPH and $ABTS^{\circ+}$ in order to recognize the efficiency of inhibitory activities. The results of $ABTS^{\circ+}$ test showed that TBHQ and α-tocopherol have more AOA than both studied essential oils. Considering the more activity of $ABTS^{\circ+}$ and the differences between their mechanisms, it can be concluded that the BPEO has lower potential for eliminating the free radicals than ZMEO [23].

Hydrogen peroxide scavenging activity

Hydrogen peroxide has a key role in biological beings, because it can enter the cell membrane and therefore it can be toxic for cells, and it may increase hydroxyl radicals in cells [25]. Also, hydrogen peroxide can be formed *in vivo* by most of enzymes such as superoxide dismutase [24].

Hydrogen peroxide can be decomposed into water and phenolic compounds can accelerate the conversion reaction H_2O_2 to H_2O by their electron donating properties. On the other hand, hydrogen peroxide can produce the very active hydroxyl radicals in presence of iron ions [26].

As it is obvious from Table 3, both studied EOs have lower antioxidant activities than TBHQ, and again ZMEO showed more antioxidant activity than BPEO. In general, antioxidants can inhibit hydrogen peroxide through three ways: inhibiting peroxide directly, reacting with intermediate compounds formed by enzymes and peroxides, and inhibiting the activity of peroxidase enzyme by binding peroxide to itself [27].

Fe-reducing power assay

In order to qualifying the antioxidant activity in spectrometry, it is dedicated some period of reaction to forming or disappearing special spectrum in an especial concentration, and the rate of absorption reduction is determined in all tests defining the antioxidant activities unless the reducing power test. The percentage of inhibition or eliminating the probe is determined by the following formula:

$$\left(1-\frac{A_{\text{Sample}}^{\lambda_{\text{Activity Assay}}}}{A_{\text{Control}}^{\lambda_{\text{Activity Assay}}}}\right)\times 100$$

Where A is absorption and $\lambda_{\text{Activity assay}}$ wavelength.

As it was stated, it is measured the rate of increase in absorption in this test, and the index of $RP_{0.5\ AU}$ is used to determine the reducing power which is equal to the rate of concentration by mg/ml or µg/ml, that is 0.5 absorption unit at 700 nm [11].

Fe-reducing power of a compound may be considered as an important index for evaluating the AOA of activity compounds. Presence of reducing agents result in reduction of complex Fe^{3+}/ferricyanide to ferrous form that is recognizable at 700 nm. In this test, the yellow color of solution will turn into green and blue [14].

As it is obvious from Table 4 and Figures 4 and 5, both essential oils showed lower reducing activity than TBHQ, and the essential oil of ZMEO showed more antioxidant activity than BPEO, like other results in pervious assays.

Effect of ZMEO and BPEO on oxidative stability of linseed oil

In order to study the effect of two EOs on the stability of linseed oil (containing higher ω-3 fatty acid in vegetable sources) at 60˚, two indices of PV and TBA values were measured (after heating at 60˚ for 15 days). In general,

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the PV indicates the total content of hydroperoxide and oxygen peroxide in fats or materials having fat.

TBA test is used since 40 years ago to detect the oxidation of fats. In this method, the amount of malondialdehyde (MDA) which is formed by secondary oxidation of initial carbonyl compounds such as non-2-enal is measured. MDA can react with thiobarbitoric acid results a pink pigment with the maximum absorption at 532-535 nm [34].

According to the Figures 6 and 7 all treatments had good effects in reducing the peroxide value compared to the control. But, all treatments selected for both essential oils had lower antioxidant activities as compared with BHT and TBHQ in concentrations of 200 and 20 ppm, respectively. On the other hand, all treatments of ZMEO 600 (ppm), ZMEO 1000 (ppm), BPEO 600 (ppm) had better antioxidant activity than BHT100 and showed such activity as the activity of TBHQ100 and all treatment except BPEO 1800 (ppm) did not show remarkable effect and follow that peroxidation peroperties.

The statistical results in TBA value test in 15th day (Figures 8 and 9) showed that TBHQ20 (ppm) had the best effect on reducing of TBA value, then BPEO600 and BHT200 had the same effect and ZMEO after these treatments contained the best effect. In a study by Van Ruth *et al*., (2001) showed that methanolic extracts of soybean seeds reduced formation of primary oxidation products up to 30% and secondary lipid oxidation products up to 99%, resulting in significantly increased oxidative stability of linseed oil [35]. Bera *et al*., (2004) used ethanolic extract of ajowan (*Carum copticum*) for stabilizing linseed (flaxseed), the results in this study indicated this extract with 0.025 g/g of oil concentration could be prevented peroxide formation [36].

In the present study, antioxidant activity of ZMEO and BPEO was investigated by 5 tests. The results from the tests showed that both essential oils showed good potential in antioxidant activities. The considerable point is that even though ZMEO had better activity than BPEO in first 4 tests, both essential oils showed the same effect in oil stability for linseed oil in same concentration of 600 ppm.

Conclusion

The results obtained in this work indicated that ZMEO and BPEO are good sources of natural antioxidants however antioxidant activity of ZMEO was higher than BPEO in 4 first tests, but our study in linseed oil by measurement of PV and TBA values demonstrated that both of EOs had the same antioxidant activity in concentration of 600 ppm, both of EOs will be used as natural antioxidants in linseed oil as a good replacement in concentration of 600 ppm with BHT (100 ppm) and TBHQ (10 ppm) as synthetic antioxidants.

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