An Investigation on the Antioxidant Activities of *Hyssopus officinalis* L. and *Echinacea purpurea* L. Plant Extracts in Oil Model System

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Abstract

Background: Nowadays, there's a growing demand for the natural antioxidants due to the harmful effects of synthetic antioxidants. Therefore, introduction of new natural antioxidants (especially those with plant origin) is very important.

Objective: The present study explores the chemical constitution and antioxidant activity of leaf extract of *Hyssopus officinalis* L. and extract of aerial parts of *Echinacea purpurea* L., as two valuable natural antioxidants in soybean oil.

Methods: Total phenolic content of the water extract of *Hyssopus officinalis* L. and *Echinacea purpurea* L. were determined by Folin–Ciocalteu method. The antioxidant activity (AOA) of these two extracts were evaluated with DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and beta carotene bleaching (BCB) methods. Furthur, the oven tests including peroxide and thiobarbituric acid values were done at 70º C in soybean oil system.

Results: Total phenolic content of *Hyssopus officinalis* L. and *Echinacea purpurea* L. were 200 and 60 mg/g phenolic components (galic acid equivalent), respectively. In DPPH$^{\bullet}$ test, EC_{50} value of *Hyssopus officinalis* L. and *Echinacea purpurea* L. were 35.6± 4.7 and 123.0± 10.9 ppm, respectively. In the oven test (in soybean oil), AOA of all concentrations of HOE was comparable to BHT and BHA at the concentration of 100 ppm. AOA of EPE was comparable to BHT at concentrations of 100 and 200 ppm, but lower than that of BHA.

Conclusion: In all three DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and beta carotene bleaching tests, the antioxidant activity of *Hyssopus officinalis* extract (HOE) was greater than that of *Echinacea purpurea* extract (EPE). The antioxidant activity of both extracts improved with increase of the concentration. Further, HOE and EPE were able to reduce the oxidation rate of soybean oil under conditions of the oven test at 70° C. Thus, these two extracts could be appropriate natural alternatives to synthetic antioxidants.

Keywords: *Hyssopus officinalis* L., *Echinacea purpurea* L., Extract, Antioxidant activity, DPPH$^{\bullet}$

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Introduction

There is a growing interest in studies of natural additives as potential antioxidants. Many sources of antioxidants of plant origin have been studied in recent years. Among these the antioxidant properties of many aromatic plants and spices have shown to be effective in retarding the process of lipid peroxidation in oils and fatty foodstuffs and have gained the interest of many research groups [1]. A great number of aromatic, spicy, medicinal and other plants contain chemical compounds exhibiting antioxidant properties [2]. Natural antioxidants are primarily plant polyphenolic compounds that can be obtained from different plant parts. Plant phenolic compounds are multifunctional and can act as reducing agents (free radical terminators), metal chelators, and singlet oxygen quenchers. Studies have shown that polyphenol-rich foods correlates with a wide range of physiological properties, such as antioxidant, anti-microbial, antimutagenic, anti-allergenic, antischemic, anti-inflammatory, immunomodulatory and antiplatelet effects [3]. Several epidemiological studies have indicated that a high intake of plant products is associated with a reduced risk of a number of chronic diseases, such as atherosclerosis and cancer [4]. These beneficial effects have been partly attributed to the compounds which possess antioxidant activity. The major antioxidants of vegetables are vitamins C and E, carotenoids, and phenolic compounds, especially flavonoids [4]. These antioxidants scavenge radicals and inhibit the chain initiation or break the chain propagation (the second defense line). Vitamin E and carotenoids also contribute to the first defense line against oxidative stress, because they quench singlet oxygen [4]. Currently, there is a strong debate about the safety aspects of chemical preservatives since they are considered responsible for many carcinogenic and teratogenic attributes as well as residual toxicity [5]. Therefore, the identification and study of novel compounds characterized with antioxidant activity from natural sources is an important strategy to improve human health condition and life quality [6]. Natural antioxidants, due to the presence of organic compounds containing phenolic or amine groups, are stable to autoxidation [7]. Generally, nowadays there's a growing concern to antioxidant activity of essential oils and plant extracts.

"Zoofa" and "Sarkhargol" are the Persian names for *Hyssopus officinalis* L. and *Echinacea purpurea* L., respectively. *Hyssopus officinalis* L. is a medicinal plant belonging to the Labiatae family. This plant is widely cultivated in south and middle parts of Europe and also in France, Russia, Italy, Iran and Spain [8]. The essential oil of *Hyssopus officinalis* L. has anti HIV effects [9] and also antibacterial and antifungal activities [8]. This plant has shown inhibition on five microorganisms including *Escherichia coli, Listeria monocytogenes* and *Staphylococcus aureus* [10]. The essential oil of *Hyssopus officinalis* L. inhibited the growth of two infectious fungi *Pyrenophora avenae* and *Pyricularia oryzae,* completely and it also had acceptable inhibiting effects on other fungi [11]. In medicine, it is used as an anti-inflammatory and anti-spasmodic agent. It can be used in treatment of high blood pressure and diabetes [12]. In traditional medicine, *Hyssopus officinalis* L. is advised in treatment of fever and rheumatism [9]. In food industry, it's used as a flavoring agent and in formulation of different kinds of sauces [8]. The chemical composition of the oil of *Hyssopus officinalis* L. has been studied in different parts of the world and the main

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component are reported to be iso-pinocamphone and pinocamphone [13].

Purple Coneflower (*Echinacea purpurea* L.) is an important medicinal plant belonging to the family *Asteraceae*. Its origin is South America and it grows greatly in the northern part of Missouri River. *Echinacea purpurea* L. is an important medicinal plant. The aerial part and root of *Echinacea purpurea* contains valuable substances which used in producing medicine for influenza. They're also immunostimulant and increase production of immunoglubolin G [14]. In Europe, among all of different species of *Echinacea*, *Echinacea purpurea* is the most common for treatment of cold [15]. Consumption of *Echinacea purpurea* decreases the probability of intestine cancer [16]. The hole extract of *Echinacea purpurea* can strongly inhibit the growth of *Candida albicans* and *Saccharomyces cerevisiae* [17]. Root extract of *Echinacea purpurea* can entrap hydroxyl radicals. This antioxidant mechanism is described as elimination of free radicals and chelating metal ions [18]. Effective compounds of *Echinacea purpurea* are alkylamids, polysacharides, glicoproteins and chicoric acid [15]. Chicoric acid is the main phenolic compound with antioxidant effect in *Echinacea purpurea* [19]. Phenolic compounds like caffeic acid and chlorogenic acid are the most efficient naturally occurring antioxidants. As the result of the presence of chicoric acid in leaf extraction of *Echinacea purpurea*, this specie has the most antioxidant effect among all other species [15].

Regarding the medicinal effects of *Hyssopus officinalis* L. and *Echinacea purpurea* L. we want to determine the antioxidant activity of these two plants. To the best of our knowledge, the antioxidant activity of *Hyssopus officinalis* L. and *Echinacea purpurea* L. originating from Iran has not been reported yet, hence, our results can be evaluated as the first report in this category.

The aims of this work were: (i) to determine the total phenolic content of HOE and EPE by using Folin-Ciocalteau method, (ii) to evaluate the AOA of these two extracts by using the 2,2'-diphenyl 1-picrylhydrazyl (DPPH) free radicals scavenging, 2,2'-Azino-di-3-ethylbenzthiazoline sulphonate (ABTS) radical scavenging and β-carotene bleaching (BCB) methods, (iii) and to determine the AOA of HOE and EPE in crude soybean oil by measuring of peroxide and thiobarbituric acid values (oven test).

Materials and Methods

Materials

Water extracts of leaves of HO and aerial parts of EP were obtained from Institute of Medicinal Plants Research in Karaj, Iran. Folin-Ciocalteu phenol reagent and ethanol were purchased from Merck (Darmstadt, Germany); 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene from Sigma–Aldrich (MO, USA) and 2, 2'-Azino-di-3-ethylbenzthiazoline sulphonate (ABTS) from Fluka (Germany). All other reagents used had the highest analytical grade.

Total Phenolic Content

Total phenolic content of the HOE and EPE were determined using Folin–Ciocalteu colorimetric method [20]. The calibration curve was used to determine the corresponding gallic acid concentration of the samples. Values were reported in gallic acid equivalents (GAE) using units of mg/g.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Free Radical Scavenging Activity

The hydrogen atom or electron- donation ability of the HOE and EPE were measured

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from the bleaching of the purple- colored ethyl acetate solution of DPPH•. This spectrophotometric method was carried out according to Ayoughi et al., 2011 [21]. All measurements were done in triplicate and the average of results was reported. The antiradical activity of the extract was calculated according to the following formula [1].

$\%\ RSA = [1 - ((A_{Control} - A_{sample}) / A_{Control}) \times 100]$

where $A_{Control}$ is the absorbance of the control at t = 0 min, A_{Sample} is the absorbance of the sample at t and RSA is the radical scavenging activity. Extract concentration providing 50 % inhibition (EC_{50}) was calculated from the graph plotting percentage of remaining DPPH• against extracts concentrations.

ABTS Free Radical Scavenging Activity

ABTS free cation radical is more active than DPPH free radical, and its reaction with the antioxidant is done completely in about one minute. The radical scavenging activity is reported according to L-ascorbic acid equivalent antioxidant capacity (AEAC) [22, 23]. Preparation of stable ABTS cation radical was done as follows: a 7 mM solution of ABTS in distilled water was made and then the concentration of this solution was decreased to 2.45 mM by potassium per sulfate solution. This solution was then kept in a dark place for 12-16 hours [22]. After the mentioned time, the solution was diluted 50 times by pure ethanol. Then 1 ml of this solution was mixed with 10 ml of antioxidant sample and the absorbance was determined after 15 minutes at 734 nm for all samples. Blank was prepared by adding water instead of antioxidant and the absorbance was measured in the first moment and after 15 minutes. The inhibition percentage of ABTS radical cation by the samples was calculated according to following formula.

$\%I = (A_{b(15)} - A_{s(15)})/A_{b(15)}) \times 100$

Where A_b is the absorbance of blank at t= 15 min, A_s is the absorbance of sample at t= 15 min and I is the inhibition percentage of the studied extracts [2, 23].

β-carotene Bleaching Assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation [21]. The test was done as previously reported by Ayoughi et al., 2011, with some modifications.

Effect of HOE and EPE on Soybean Oil Oxidation

The HOE and EPE were added to crude soybean oil at 200, 400, 600 and 1000 ppm. AOA of essential oils were compared with synthetic antioxidants including BHA, BHT at 100 and 200 ppm. The oven test method at 70 °C was used to check crude oil stability. Oxidation was periodically assessed by the measurement of peroxide (PV) and thiobarbituric acid (TBA) values at 0th, 5th, 10th, 15th and 20th days of storage according to the AOCS methods [24, 25]. All experiments were performed in triplicate and results were averaged.

Statistical Analysis

All tests were performed in triplicate. Results are presented as mean± standard deviation of three independent determinations. All statistical analyses were carried out by MS Excel software and SAS software using analysis of variance (ANOVA) and differences among the means were determined for significance at $p \leq 0.05$ using least significant

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differences (LSD) test.

Results

Amount of Total Phenolic Compounds

Since we're investigating the antioxidant properties of HO and EP extracts, and due to the fact that phenolic compounds in plants are responsible for antioxidant properties exhibited by them [26, 27], total phenolic contents were measured. TPC of the water extract of HO and EP were determined by Folin–Ciocalteu method. According to our results, HO and EP water extracts contain 20% and 6% phenolic component, respectively. Comparing the results of TPC test in the mentioned extracts, it can be concluded that higher phenolic content of HO extract leads to higher antioxidant activity of HO than EP.

DPPH Radical Scavenging Activities of the Extracts

Fig. 1A and B show the effect of different concentrations of HOE and EPE on scavenging rate of $DPPH^{\bullet}$, respectively. There is a direct correlation between remaining DPPH free radicals and extracts concentrations. The remaining DPPH free radicals increased with increasing the extract concentrations. Higher AOA activity was observed in lower EC_{50} values. EC_{50} values of HOE and EPE have been compared with other extracts, essential oils and BHT. HO exhibited higher radical scavenging activity (EC_{50} = 35.6 ± 4.7 ppm) than EP (EC_{50} = 123.0 ± 10.9 ppm).

ABTS Assay

By $ABTS^{\bullet+}$ method, antioxidant activity of carotenoids, phenolics and some plasma antioxidants can be measured through decolorization of $ABTS^{\bullet+}$ and thus, measuring the reduction of the radical cation as the percentage inhibition of the absorbance at 734 nm [22]. Different concentrations of HOE and EPE were tested with $ABTS^{\bullet+}$. The AOA of different concentrations of HOE and EPE equivalent to L-ascorbic acid antioxidant capacity are shown in Fig. 2A and B, respectively. With increase in the concentration of these extracts, the cation radical scavenging activity increased which could be due to higher phenolic content at higher concentrations. As it's shown in the figures, HOE has exhibited higher AOA than EPE. HOE at concentration of 100 ppm acted almost like EPE at concentration of 1000 ppm. This could be again, a result of higher phenolic content of HOE. For HOE, concentration of 3750 ppm had the inhibition percentage of 89.58 ± 0.84, and EPE at concentration of 2500 ppm had inhibition percentage of 83.94 ± 1.39. Due to color interference of extracts with $ABTS^{\bullet+}$ solution color, higher concentrations could not be examined.

β - carotene Bleaching Assay

In β-carotene bleaching method, by measuring oxidation products of linoleic acid, its degree of oxidation is determined. The oxidation products are lipid hydroperoxides, conjugated dienes, and volatile by-products that attack beta carotene and bleaching its yellow color [28]. Six different concentrations of HOE and EPE were prepared. BHA at two concentrations was used as positive control. The results are compared and shown in Fig 3. Here again, AOA increased with the increase of concentration. HOE and EPE at concentration of 750 ppm exhibited maximum AOA, with higher AOA of HOE, as a result of its higher phenolic content. Higher concentrations of these extracts were not examined due to color interference. BHA at concentration of 200 ppm showed the best AOA among all ($p < 0.05$).

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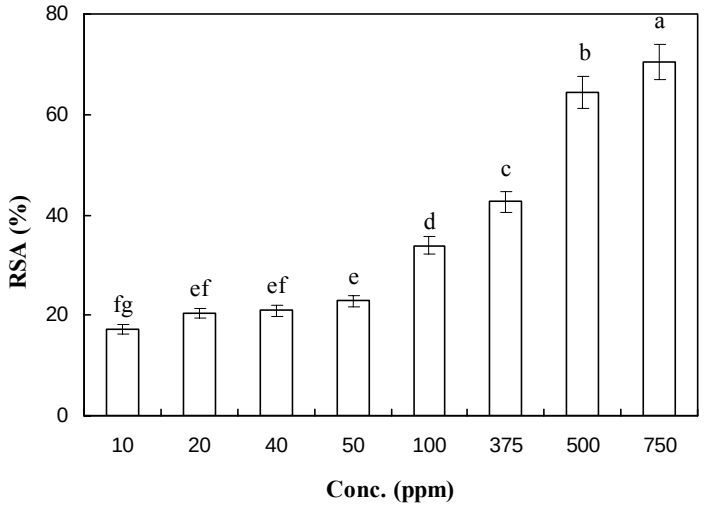

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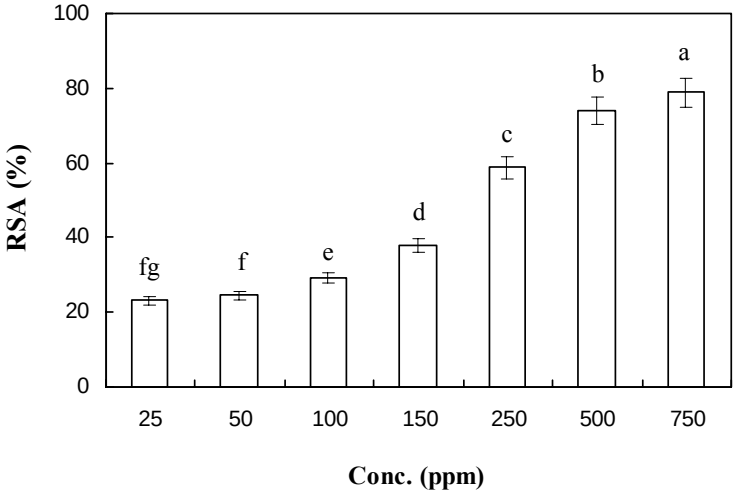
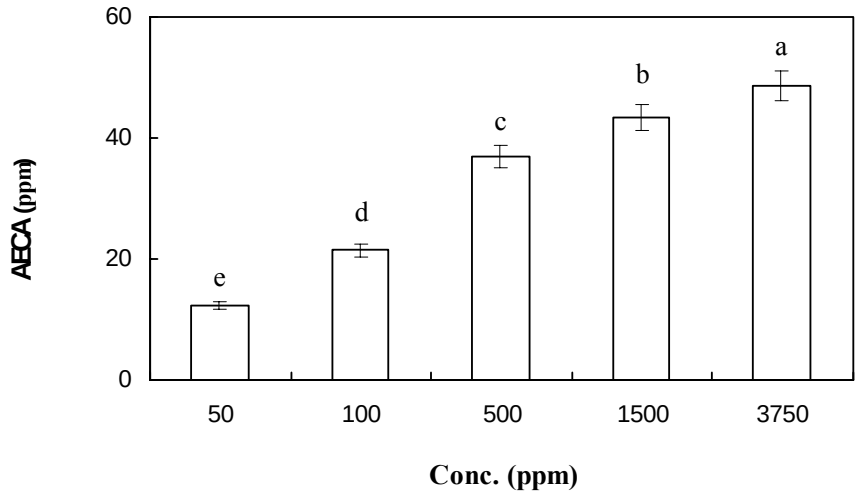


B

Fig. 1- Effect of different concentrations of HOE and EPE on RSA (A and B, respectively)



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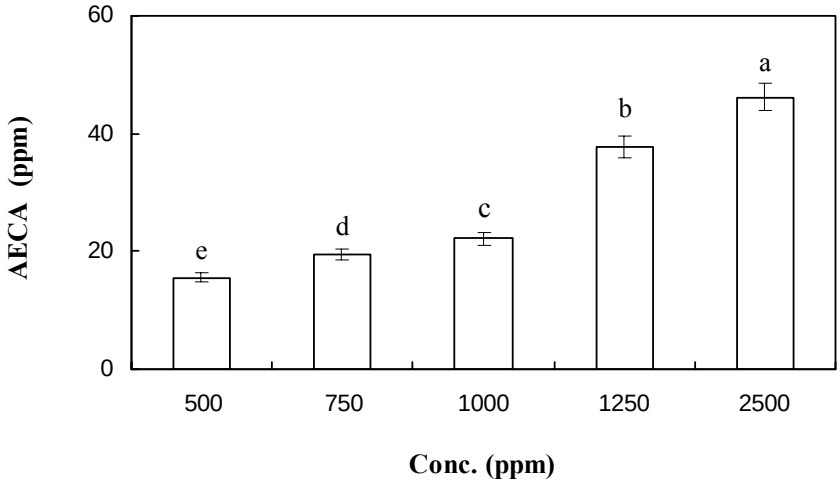

B

Fig. 2 - Antiradical activity of HOE and EPE determined by $ABTS^{\bullet+}$ (A and B, respectively) method

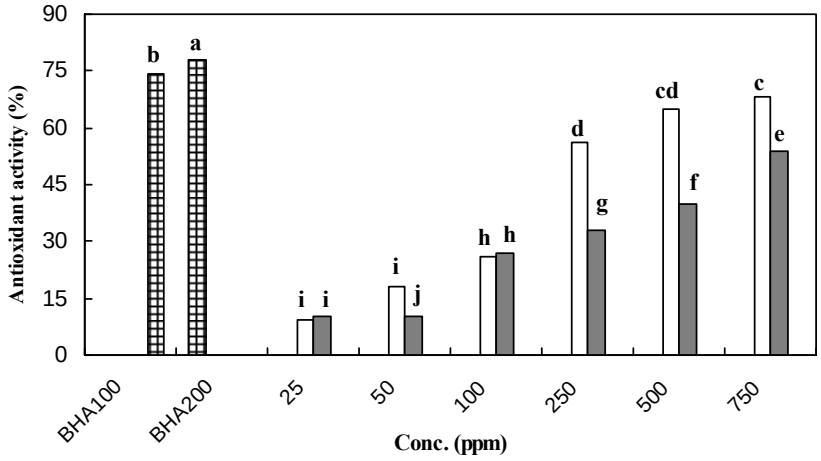

Fig 3 - Antioxidant activity of different concentrations of HOE (white column) and EPE (gray column) determined by β- carotene bleaching method

Effect of HOE and EPE on Soybean Oil Oxidation

Peroxides are primary products of lipid oxidation. The more the unsaturation degree of oil, the more is proneness to oxidation. Fig 4 shows changes in peroxide value in soybean oil of investigated samples at 70 °C. PV was periodically assessed at time intervals of 5 days during 20 days of storage. During the oxidation, PV increased to a certain point and then it decreased due to the degradation of peroxides into secondary oxidation products like malonaldehyde, which caused a bad smell in oils. Thus, changes in secondary oxidation products were measured by TBA test, at time intervals of 5 days (Fig. 5).

All samples containing 200-1000 ppm of HOE were more stable on heating at 70 °C than the control when assessed by the change in peroxide (Fig. 4) and TBA (Fig. 5) values. AOA of HOE was comparable to all concentrations of BHT, BHA at the concentration of 100 ppm and lower than BHA at the concentration of 200 ppm. The antioxidative effect of HOE showed a correlation with concentration but this correlation was not linear, which may be due

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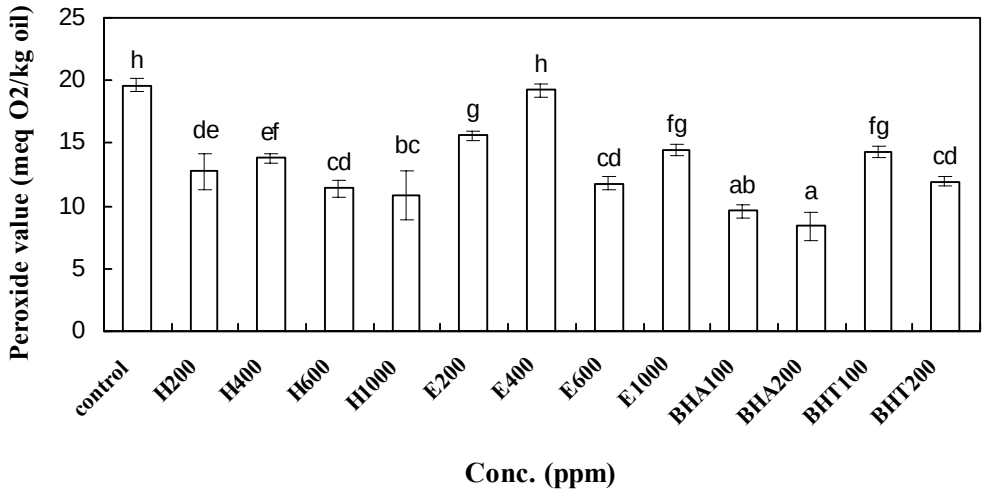

Fig 4 - Effect of HOE and EPE on soybean oil oxidation expressed as peroxide value during storage at 70 °C

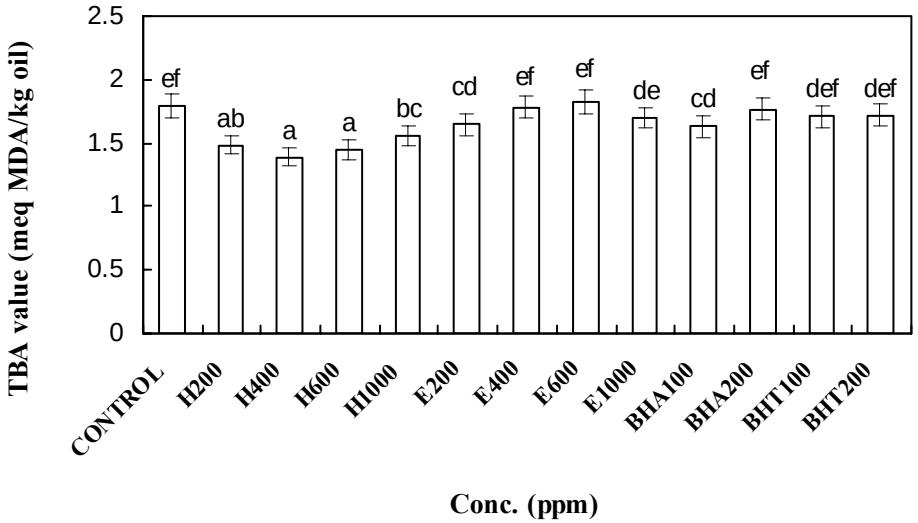

Fig 5- Effect of HOE and EPE on soybean oil oxidation expressed as thiobarbituric acid value during storage at 70 °C

to interference of other components of the extract or some mixing problems. At a concentration of 600 and 1000 ppm, the AOA was not significantly different from that of the BHA at concentration of 100 ppm, and BHT at concentration of 200 ppm.

Most treatments with EPE level added at 200-1000 ppm were more stable on heating at 70 °C than the control, when assessed by the changes in peroxide (Fig. 4) and TBA (Fig. 5) values. In PV test, AOA of EPE was comparable to BHT but lower than BHA. The antioxidative effect of EPE didn't show a direct correlation with concentration and at a concentration of 600 ppm was equal to BHT at 200 ppm and at the concentrations of 200 and1000 ppm it was equal to BHT at 100 ppm. In oven test generally, AOA of HOE at 1000

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ppm was equal to that of BHA at 100 ppm and BHT at 200 ppm, and AOA of EPE at 600 ppm was equal to that of BHT at 200 ppm. These results showed a correlation between peroxide and TBA values. However, HOE had higher AOA than EPE in soybean oil, as a result of its higher phenolic content.

Discussion

Differences in chemical compositions of the extracts obtained from different parts of the world could be due to use of various species, different climates and cultivation and storage conditions. Differences in the composition of the oils and extracts affect their antioxidant properties [9]. Chemical components of essential oils of HO and EP have been investigated in different parts of the world. Iso-pino camphone and pinocamphone are the main components of HO essential oil [13] and for EP it's been found that chicoric acid is the main phenolic compound of the plant which is made of two caffeic acid molecules [15].

The radical scavenging activity of HOE and EPE are more than activities reported by other researches. Ayoughi et al. reported EC_{50} values of *Anethum graveolens* L. and *Artemisia dracunculus* L. essential oils in 2011 [21]. These values were 2750 ±150 ppm and 3190 ± 130 ppm, respectively. In another study conducted by Fazel et al. in 2008, the AOA of *Thymus vulgaris* L. and *Satureja hortensis* L. essential oils were investigated and EC_{50} values were reported as 8900 and 5800 ppm, respectively [29]. Shahsavari et al. also reported the EC_{50} value for *Zataria multiflora* L. as 2220 ± 40 ppm, in 2008 [30]. In 2006, Zhang et al. reported EC_{50} value of 80210 ± 3410 for the essential oil of *Petroselinum crispum* L. [31]. This higher AOA of HOE and EPE can be due to their high phenolic content. HOE showed higher AOA than EPE, this was predictable, due to higher phenolic content of HOE. In addition, EC_{50} values of HOE and EPE were compared with that of BHT reported by Ayoughi (EC_{50}= 38.0 ± 1.0 ppm) [21]. HOE had better AOA in comparison with BHT; introducing it as a potent natural antioxidant.

Our results showed good antioxidant activities for HOE and EPE in a food system (crude soybean oil at $p<0.005$). These findings are comparable with the results of a study on the AOA of *Artemisia dracunculus* L. and *Matricaria chamomilla* L. essential oïl in soy bean oil [21]. This study showed that the effect of 600 and 800 ppm of *Artemisia dracunculus* L. essential oil was comparable to that of 100 and 200 ppm of BHT. *Matricaria chamomilla* L. essential oil at concentration of 800 ppm had the same effect as BHT 100 ppm on oïl oxidation. In another study, AOA of *Zataria multiflora* L. essential oil in soybean oil at the concentration of 1000 ppm was comparable to that of BHA at concentration of 200 ppm [30]. An investigation on the pistachio hull extract on retarding oil oxidation showed that at concentration of 600 ppm, this extract acted like BHA and BHT at the concentration of 200 [32]. Yasoubi et al. investigated AOA of pomegranate (*Punica granatum* L.) peel extracts in soybean oil in 2007. The extract at concentration of 500 ppm was comparable to BHA and BHT at concentration of 200 ppm [33].

Conclusion

According to the tests carried out and determining the AOA of both HOE and EPE, the study shown that HOE and EPE had antioxidant properties. HOE posed stronger AOA than EPE, due to higher phenolic content. However, EPE in some concentrations

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exhibited great AOA. Thus, these two extracts could be appropriate natural alternatives to synthetic antioxidants, having no addition limits to get optimal effects (unlike synthetic antioxidants). However, further investigations (such as effect of different food processes on the properties of these antioxidants, combination of these extracts and the probable synergistic effects and etc.) and the antioxidant activity mechanism are warranted. These studies are starting points for further application of natural antioxidants as alternatives to synthetic ones in food preparations.

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