Antioxidant Activity of *Satureja hortensis* L. Essential Oil and its Application in Safflower Oil

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Abstract

Background: It has been considered by researchers to study the possibility of replacing chemical food additives (synthetic antioxidants) by natural products (medicinal plants).

Objective: This study investigated the antioxidant properties of *Satureja hortensis* L. essential oil (SHEO) on safflower oil oxidation.

Methods: Different assays have been used to evaluate the antioxidant activity of SHEO: total phenol content (TPC), DPPH$^{\bullet}$, ABTS$^{+\bullet}$, ferric thiocyanate (FTC), β - carotene bleaching. For evaluation of SHEO effect on safflower oil oxidation, peroxide value (PV), conjugated dienes (CD), and tiobarbituric acid (TBA) indices were compared with BHT (a synthetic antioxidant).

Results: TPC of SHEO was determined to be 293.7 mg gallic acid equivalent in 1 ml of sample and IC_{50} was 0.71 mg/ml in DPPH•. 0.4 and 0.1 mg/ml of SHEO at all time (1, 5, 10, 15 min) showed the highest and lowest antiradical ABTS°+ activity (118.2 and 26.6 µg/ml AscAE =Ascorbic acid equivalent) in 15 min. In FTC and FTC-TBA, 8 mg/ml SHEO showed the highest activity. In β-carotene bleaching of 0.1-2 mg/ml SHEO, 0.1 has the minimum (%9.02), and 1 and 2 has the maximum inhibitory effects (%34.33 and %36.86 inhibitions). 4 mg/ml of SHEO had the highest inhibitory effect in the safflower oil test and that peroxide does not have significant difference with 0.1 mg/ml BHT.

Conclusion: Antioxidant activities of SHEO concentrations increased in all indices ($p< 0.05$) and various concentrations were able to slow down the oxidation process.

Keywords: ABTS$^{+\bullet}$, Antioxidant activity, β-carotene bleaching, DPPH$^{\bullet}$, FTC, Safflower oil, *Satureja hortensis* L.

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Introduction

Antioxidants are compounds that inhibit the oxidation of molecules to avoid/ delay the start or continuation of oxidation reaction. There are two main categories of antioxidants; namely synthetic and natural. Generally, synthetic antioxidants are phenolic compounds with different alkyl degrees, while natural antioxidants can be phenols (tocopherol, flavonoids, and phenolic acids), nitrogen compounds (alkaloids, chlorophyll derivatives, amino acids, and amines), carotenoids, or ascorbic acid [1, 2]. Antioxidant compounds, as agents to protect health, play an important role in food [3]. Many natural antioxidants, particularly flavonoids show a wide range of biological effects, including antibacterial, antiviral, anti-inflammatory, anti-allergic, antithrombotic, and vasodilatory actions [1]. During storage, oxidation of oils and fats in food containing fat is yet the main reason for loss of food quality despite the widespread use of various antioxidants. Widely used synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) can quite easily volatilize and decomposed in high temperatures. There are serious problems related to safety and toxicity of BHA, BHT, and tert-butylhydroquinone (TBHQ), related to their metabolites and their possible absorption and accumulation in organs and tissues. Therefore, search for useful natural antioxidant are greatly considered [2, 4].

In addition, antioxidants widely used as additives to fat, oils, and processed foods in order to prevent or delay corruption. Some spices and plants are accepted as a source of effective antioxidants [3].

Satureja hortensis L. is a medicinal herb recognized by its different effects in drug therapy and traditional medicine, and is widely used in Iran. *Satureja hortensis* L. is a plant, belongs to *Lamiaceae* family (*Labiatae*) and it is among the species with significant antioxidant effects. The most important compounds of *Satureja hortensis* L. essential oil (SHEO) are carvacrol (30 - 40%), thymol (20 - 30%), and some phenolic compounds. This plant is cultivated in various parts of Iran and has different therapeutic effects including carminative properties, and is used for many gastrointestinal disorders such as abdominal cramps, nausea, indigestion, and problems with appetite. Moreover, it is useful for treating muscle pains, cramps, and infections [5].

Satureja hortensis L. extract have different activities including antibacterial, antifungal, antioxidant, and analgesic effects [6]. In recent studies, antioxidant activity of *Satureja hortensis* L. extract has been determined [7].

In recent years, there is an increasing tendency among people to ensure that healthy foods are more similar to safflower oil. The seeds of safflower have approximately 27-32% oil and it is a rich source of vitamin E. The linoleic acid of safflower oil is 55-81%, which is higher than all available vegetable oils [8, 9].

Study on the use of natural preservatives in foods and replacing chemical preservatives with natural ones are increasing. In this regard, consumers' awareness about risks of chemical preservatives and tendency toward using natural products is rising. The aim of the current study, in addition to complete the

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previous studies, is the evaluation of SHEO as a natural preservative by 4 methods: $DPPH^{\circ}$, $ABTS^{\circ+}$, ferric thiocyanate (FTC) and *β*-carotene bleaching in safflower oil model system (containing higher ω-6 fatty acid in vegetable sources [8]) .for the first time to the baseofour knowledge.In addition, to determine the antioxidant capacity of SHEO byperoxide, conjugateddienesand thiobarbitoric acid values and compared with BHT.

Material and Methods

Materials

We purchased 2, 2′-diphenyl 1-picrylhydrazyl ($DPPH^{\bullet}$), 2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulfonate) diammonium salt ($ABTS^{\bullet+}$), β-carotene, linoleic acid, and cyclohexane from Fluka BioChemika (Germany). Chloroform, ethanol, acetone, sodium thiosulfate, starch, acetic acid, ammonium thiocyanate, iron (II) chloride, trichloroacetic acid (TCA), 2-tiobarbituric acid (TBA), Tween 20, ascorbic acid, Folin-Ciocalteu reagent, gallic acid, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), potassium iodide, potassium iodate, phosphate-buffered saline (PBS), potassium persulfate, and hydrogen peroxide were from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

SHEO was obtained from Institute of Medicinal Plants and Natural Products Research, Karaj, Iran and kept at −4 °C until use. Safflower seeds, variety of Esfahan 28, were obtained from Institute of Seed and Plant Breeding, Karaj, Iran.

Methods

Determination of total phenolic compounds (TPC)

Total phenolic content of SHEO was determined with Folin-Ciocalteu reagent, according to the method described by Waterhouse, 2002 [10]. Total phenolic content in SHEO was determined in mg of gallic acid equivalent (GAE) using an equation obtained from the standard gallic acid graph (R^2: 0.9994):

GAE = (Abs-0.0254)/0.0012

Briefly, 20 µl of the sample was transferred to a flask, and 1.58 ml ultra-pure water was added, subsequently 100 µl undiluted Folin-Ciocalteu reagent was added to the flask contents. After 5 min of shaking, 300 µl of saturated Na_2CO_3 was added and the mixture was kept at room temperature for 2 hour. Finally, the absorbance was measured at 765 nm and compared to the gallic acid calibration curve.

Antioxidant activity

$DPPH^{\bullet}$ assay

The ability of SHEO to scavenge stable 2, 2′-diphenyl 1-picrylhydrazyl radical ($DPPH^{\bullet}$) was assessed by spectrophotometer. Two mL of various concentrations of the samples (0.3–3 mg/ml) in ethanol were added to 1 ml of a 0.2 mM ethanol solution of DPPH•. After 1 h, the decrease in absorbance at 517 nm was determined by Scinco spectrophotometer (Seoul, South Korea) for all samples. The absorbance of the ethanol solution DPPH radical without antioxidant was measured as control. All determinations were performed in

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triplicate and results were averaged. The inhibition percentage of the DPPH radical by the sample was calculated according to the following formula [11]:

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

Where $A_{Control}$ is absorbance of the control and A_{Sample} is absorbance of the sample after 1 hour. Essential oil concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting percentage of radical scavenging activity (% RSA) against SHEO concentration.

$ABTS^{+\bullet}$ assay

Determination of $ABTS^{\bullet+}$ radical scavenging was carried out as described by Re *et al.* 1999 [12]. The ABTS•+ radical was generated by reacting a 7-mmol/l ABTS aqueous solution with $K_2S_2O_8$ (2.45 mmol/l, final concentration) in the dark for 12–16 h at ambient temperature, and adjusting the Abs 734nm to 0.7 (7 ± 0.02) with ethanol. The samples were diluted, such that, when 10 µl of the sample was added to 1 ml $ABTS^{\bullet+}$, it resulted in a 20–80% inhibition of the blank absorbance. After addition of 1 ml $ABTS^{\bullet+}$ solution to 10 µl of the sample, the absorbance at 734 nm was recorded 1 min after initial mixing and subsequently for 15 min in total. The results are expressed as the ascorbic acid equivalent antioxidant capacity (AscAE, µg/ml Asc) at minutes 1, 5, 10, and 15 and as the relative antioxidant activity.

Ferric thiocyanate (FTC) method

The antioxidant activity of SHEO was determined according to the ferric thiocyanate method in linoleic acid emulsion as described by Inatani *et al*. 1983; Larrauri *et al*. 1997; and Zainol *et al*. 2003 [13 - 15] with slight modifications. In this method, peroxide formation occurred during linoleic acid oxidation. These compounds oxidized Fe^{2+} to Fe^{3+}. The latter ions form a complex with thiocyanate and this complex has the maximum absorbance at 500 nm.

A mixture of 0.5 ml of various concentrations of SHEO in absolute ethanol with 0.5 ml of 2.51% linoleic acid in absolute alcohol, 1 ml of 0.05 M phosphate buffer (pH 7.0), and 0.5 ml of water was placed in a vial with a screw cap and then placed in an oven at 40 °C in the dark. In the control, the sample was replaced with an equal volume of ethanol. Then, 4.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate was added to 0.1 ml of this solution. Precisely three minutes after addition of 0.1 ml of 0.02 M ferrous chloride in 3.5% hydrochloride acid to the reaction mixture, the absorbance of the red color developed was measured at 500 nm every 24 hours until absorbance of the control reached maximum.

***β*-carotene bleaching assay**

The antioxidative activity of SHEO was evaluated using a β-carotene-linoleic acid (linoleate)modelfollowinga modification of the procedure describedbyOthman *et al.* 2007[16].

Briefly, 5 ml of β-carotene (0.2 mg/ml) dissolved in chloroform was pipetted into a small round-bottom flask containing 20 mg of linoleic acid and 200 mg of Tween 20. After removing the chloroform by a rotary

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evaporator (Unimax 1010, Heidolph, Germany), 50 ml of hydrogen peroxide (H_2O_2) was added to the flask with vigorous stirring. Aliquots (2 ml) of the prepared emulsion were transferred to a series of tubes containing 5 µl of SHEO or BHA and BHT. Each type of sample was prepared in triplicate. The test systems were placed in a water bath at 50 °C for 120 min. The absorbance of each sample was measured at 470 nm, immediately after sample preparation (t=0 min) and at 20-min intervals until the end (t=120 min) of the experiment.

Degradation rate (DR) was calculated according to first order kinetics, using the following equation based on Velioglu *et al.* 1998; and Amarowicz *et al.* 2004 [1, 17]:

$DR= \ln (a/b) \times 1/t$

Where in is the natural log, **a** is the initial absorbance (470 nm) at time 0, **b** is the absorbance (470 nm) at 20, 40, 60, 80, 100, or 120 min; and **t** is the time (min).

Antioxidant activity (AA) was expressed as percentage of inhibition relative to the control, using the following formula:

$\%AA= ((DR_{control} - DR_{sample\ or\ standard}) / DR_{control}) \times 100$

Tiobarbituric acid (TBA) method

The method provided by Zainol *et al.* 2003 [15] was adopted. An amount of 2 ml of 20% trichloroacetic acid (TCA) and 2 ml of 0.67% thiobarbiturate acid (TBA) aqueous solutions were added into 1 ml of the sample solution, prepared and incubated as above. The mixture was placed in a boiling water bath for 10 minutes. After cooling, it was centrifuged at 3000 rpm for 20 minutes and absorbance of the supernatant was measured at 532 nm.

Conditioning of safflower seeds and extraction of oil

Safflower seeds were placed in the room for a period of 2-3 days, during which the relative humidity reaches about 45 percent.

After drying and cleaning of seeds, they were ground. Retrieved safflower seeds with specified moisture content were used for extraction with hexane solvent flooding method. Then, the ground safflower seeds deposited into cartouche and left in container with hexane solvent. In the following, the container was placed into the incubator under continuous shaking and constant temperature of 15 °C for 10 hours. The mixture of solvent and oil were separated by rotary evaporator system under vacuum at 30 °C [18]. Extracted safflower oil was kept in -24 °C for the next tests.

Effect of SHEO on safflower oil oxidation

The SHEO added to safflower oil at 0.5, 1, 2, and 4 mg/ml. Synthetic antioxidants (BHA, BHT) at concentrations of 0.1 and 0.2 mg/ml were used for comparison. The oven test method at 60 °C was used to check stability. Oxidation was periodically assessed by measurement of the peroxide value (PV) at days 0, 2, 4, 6, 8, 10, and 12 of storage according to the AOCS method [18] and measurement of conjugated diene hydroperoxides and TBA values of the samples at the same days of storage according to the method described by Senevirathne *et al.* 2006 [19]. A control sample was prepared

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under the same conditions without adding any antioxidant. All determinations were performed in triplicate and results were averaged.

Statistical Analysis

Experimental data was analyzed using one-way analysis of variance (ANOVA) and significant differences among means from a triplicate analysis at $p<0.05$ were determined by Duncan's multiple range test (DMRT) using SPSS v.16.0 for Windows (SPSS Inc., Chicago) software.

Results

Total phenolic compounds

As in Table 1, total phenol content of SHEO was determined to be 293.7 ± 22.3 to mg gallic acid equivalent in one ml of the sample for the present study. According to the results, the total phenol content of SHEO is higher than that in ethanol and acetone extract of SH.

DPPH˚ assay

Figure 1 shows the decreasing trend of remaining DPPH˚ percentage in 60 minutes in the presence of different concentrations of SHEO (S-0.3 to S-3, is concentrations of SHEO 0.3 to 3 mg/ml). As can be seen, as the SHEO concentration increased, DPPH˚ percentage decreased.

Figure 2 shows the relationship between DPPH radical scavenging activity (RSA) and concentration of 0.3- 3 mg/ml of SHEO. As can be seen, as the SHEO concentration increased, DPPH radical scavenging activity increased. In addition, the results showed that the concentration of 3 mg/ml of SHEO had the highest radical scavenging activity. Using this test, IC_{50} of SHEO was determined as 0.71 ± 0.01 mg/ml (Table 2).

ABTS°$^{+}$ assay

Table 3 shows the radical cation ABTS scavenging activity of SHEO at concentrations of 0.1 – 0.4 mg/ml in comparison with synthetic antioxidant TBHQ in 0.05 mg/ml as ascorbic acid equivalent (AscAE).

Considering the antiradical activity of any concentration over time, there was a significant difference between the antiradical activity in 15 min to 1 min for all concentrations of SHEO (0.1 – 0.4 mg/ml) and synthetic antioxidant TBHQ in 0.05 mg/ml (similar capital Latin A-D).

Table 1- Comparison of total phenol content of SHEO in present study and SH extract in another research

Sample	Total phenolic content	Reference
Satureja hortensis L. essential oil	293.7 ± 22.3‡	Present study
Ethanol extract of *Satureja hortensis* L.	194 ± 2.1†	[26]
Acetone extract of *Satureja hortensis* L.	112 ± 5.2†	[26]

‡ mg equivalent gallic acid in ml essential oil
† mg equivalent gallic acid in g dry matter

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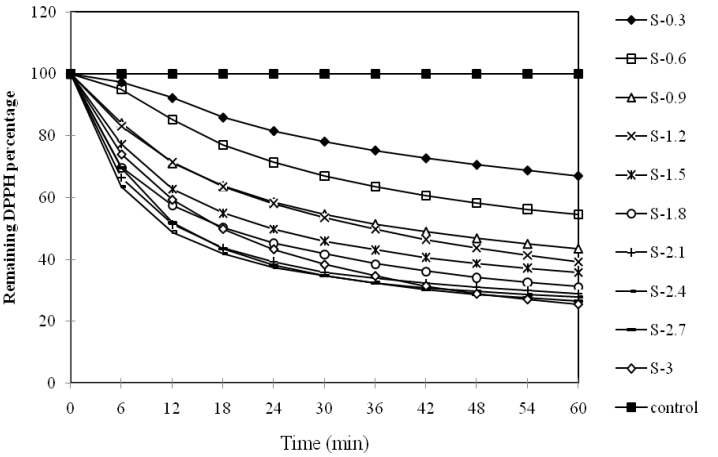


Figure 1- The decreasing trend of remaining DPPH˙ percentage in 60 minutes in the presence of different concentrations of SHEO (S-0.3 to S-3, is concentrations of SHEO 0.3 to 3 mg/ml).

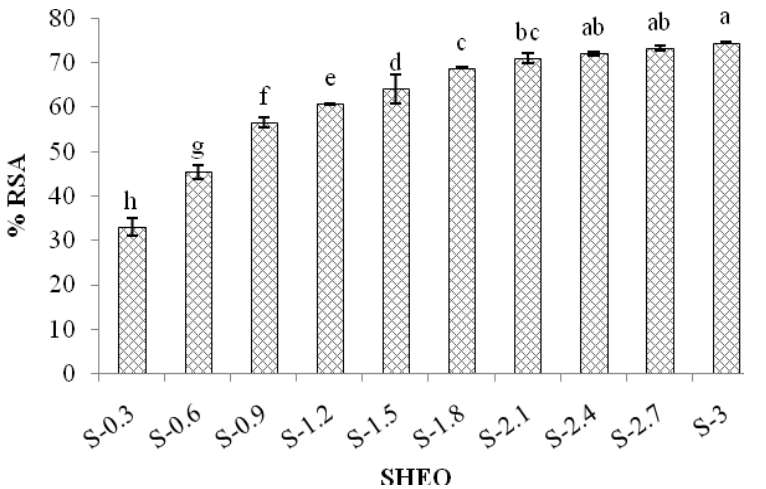


Figure 2- Relationship between DPPH radical scavenging activity (RSA) and concentration of SHEO (S-0.3 to S-3, is concentrations of SHEO 0.3 to 3 mg/ml).

Table 2- IC_{50} of SHEO and other essential oils and standards

Sample	IC_{50} (mg/ml)	Reference
Satureja hortensis L. essential oil	0.71 ± 0.01	Present study
Water extract of *Satureja hortensis* L.	2.16 ± 0.04	[7]
Zataria multiflora boiss. essential oil	2.22 ± 0.04	[29]
Petroselinum crispum L. essential oil	80.21 ± 3.41	[11]
α-Tocopherol	0.086	[11]
TBHQ	0.002	Present study

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Table 3- ABTS°$^{+}$ scavenging activity of SHEO

Sample	Concentration (mg/ml)	Ascorbic acid equivalent (µg/ml Asc) in various time (min)			
		1	5	10	15
SHEO	0.1	6.2 ± 2.8 ^{e,B}	23.6 ± 1.9 ^{f,A}	26.9 ± 2.4 ^{e,A}	26.6 ± 2.2 ^{e,A}
	0.175	15.3 ± 3.9 ^{d,B}	46.6 ± 3.0 ^{e,A}	51.7 ± 3.7 ^{d,A}	52.8 ± 4.1 ^{d,A}
	0.25	22.9 ± 1.8 ^{c,C}	63.6 ± 3.8 ^{d,B}	74.5 ± 7.3 ^{c,A}	75.7 ± 7.3 ^{c,A}
	0.325	40.8 ± 4.9 ^{b,C}	86.6 ± 3.2 ^{c,B}	101.9 ± 2.8 ^{b,A}	108.2 ± 2.4 ^{b,A}
	0.4	41.3 ± 2.3 ^{b,D}	93.1 ± 2.6 ^{b,C}	110.3 ± 2.7 ^{a,B}	118.2 ± 2.7 ^{a,A}
TBHQ	0.05	94.5 ± 0.9 ^{a,C}	107.7 ± 0.3 ^{a,B}	108.3 ± 0.3 ^{ab,AB}	108.9 ± 0.2 ^{b,A}

There is no significant difference between the similar lowercase Latin (a-j) in each column and similar capital Latin (A-D) in each row ($P<0.05$). All data is three replicates of mean ± SD

Also, among the various concentrations of SHEO, 0.4 and 0.1 mg/ml at all time 1, 5, 10 and 15 min showed the highest and lowest antiradical ABTS°$^{+}$ activity with 118.2 ± 2.7 and 26.6 ± 2.2 µg/ml (AscAE) at 15 min, respectively.

Figure 3 shows an increasing absorption trend in the presence of different SHEO concentrations in 10 days. As it is clear, from day 6, there is no difference between absorption of various concentrations of SHEO and the control sample.

In the control sample, from day one to six, the increasing absorption trend is linear and from day six to eight, the absorption gradient was increasing. Ultimately, absorption reached its maximum in day 9 and it decreased in day 10.

Similarly, the increasing absorption trend in SHEO concentrations of 1-8 mg/ml have a linear trend in the day one to six and have an upward slope from day six to ten.

Ferric thiocyanate (FTC) assay

Figure 4 shows antioxidant activity of 1-8 mg/ml of SHEO compared with 0.2 mg/ml of BHT and sample without the antioxidant on day ten in the FTC assay.

Accordingly, it was observed that the various concentrations of SHEO had lower absorption as the concentration increased, which indicate an increase in inhibiting development of linoleic acid primary oxidation products; namely peroxide. The SHEO concentration of 8 mg/ml showed the highest antioxidant activity among the concentrations evaluated.

FTC-TBA assay

The TBA assay was used for determination of antioxidant activity and inhibition of secondary linoleic acid oxidation products. Figure 5 shows antioxidant activity of SHEO at 1-8 mg/ml concentrations in comparison with 0.2 mg/ml of BHT and the control after day 10 of the FTC-TBA assay.

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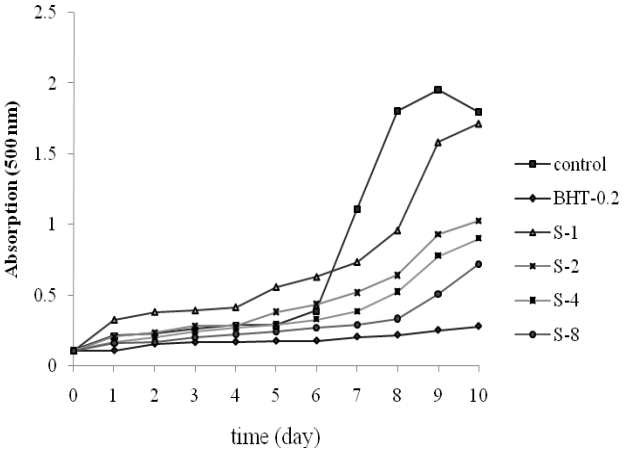


Figure 3- Increasing absorption trend in the presence of different SHEO concentration in 10 days

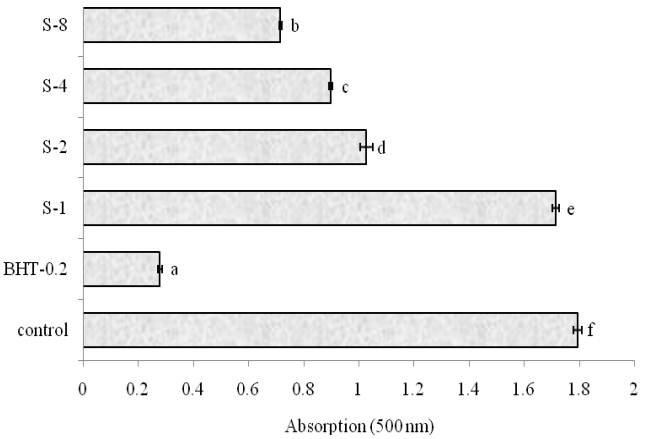


Figure 4- Antioxidant activity of 1-8 mg/ml of SHEO compared with 0.2 mg/ml of BHT and sample without the antioxidant after 10th day in the FTC assay

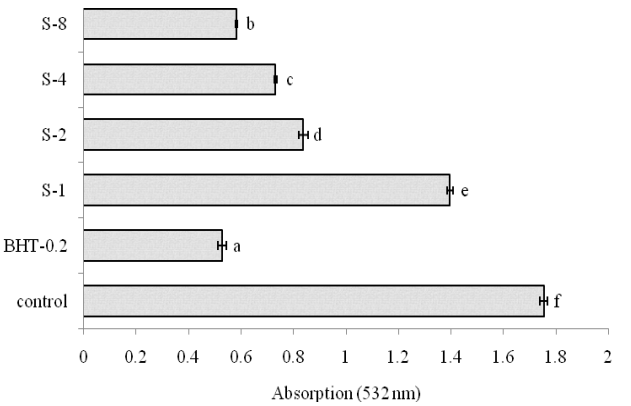


Figure 5- Antioxidant activity concentration of 1-8 mg/ml SHEO in comparison with 0.2 mg/ml BHT and control after 10th day of FTC-TBA assay

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The TBA assay is a quantitative indicator of secondary oxidation products. The results showed that antioxidant activity of SHEO on formation of secondary oxidation products, including malondialdehyde (MDA) increased with as the SHEO concentration increased from 1 to 8 mg/ml, and the highest inhibition was observed for 8 mg/ml of SHEO.

β-carotene bleaching assay

Figure 6 shows the degradation rate of 0.1-2 mg/ml of SHEO in the β-carotene bleaching assay. As can be observed, the degradation rate of β-carotene in all SHEO concentrations is higher than 0.1 and 0.2 mg/ml of BHA and BHT. Thus, the SHEO concentrations of 0.1-2 mg/ml showed lower antioxidant activity than those of 0.1 and 0.2 mg/ml of BHA and BHT. Antioxidant activity of 0.1-2 mg/ml of SHEO was evaluated as the inhibition percentage based on the ability of antioxidants to prevent lipid peroxidation and to reduce oxidation of β-carotene/linoleic acid emulsion system stimulated by heat. The activity was then compared with 0.1 and 0.2 mg/ml of synthetic antioxidants; BHA and BHT [17, 36, 37].

As shown in Figure 7, for 0.1-2 mg/ml of SHEO, 0.1 mg/ml has the minimum inhibitory effect with inhibition rate of %9.02 ± 3.07, and 1 and 2 mg/ml have the maximum inhibitory effects with inhibition rates of %34.33 ± 1.05 and %36.86 ± 1.52, respectively, and these concentrations were not significantly different.

Effect of SHEO on safflower oil oxidation

Antioxidant activities of SHEO in safflower oil are shown as peroxide, CD, and TBA values in A, B, and C parts of Figure 8, respectively.

As it is shown, the PVs, CD, and TBA values is dependent on treatment concentrations and the PVs, CD, and TBA values is reduced by increasing the treatment concentrations, and thus the antioxidant effect increased ($p<0.05$). The results showed that SHEO is capable to slow the oxidation of safflower oil under accelerated conditions (60 °C tem) and its effects is comparable with synthetic antioxidants. In addition, it was shown that there was no significant difference between SHEO concentrations of 1 and 2 mg/ml and the BHT concentration of 0.2 mg/ml in inhibitory effect on peroxidation of safflower oil. Also, no statistically significant difference was observed between 4 mg/ml of SHEO and 0.1 mg/ml of BHT.

All SHEO treatments showed inhibitory effect against formation of conjugated dienes (CD) and no significant difference was observed between the antioxidant effects of 2 and 4 mg/ml of SHEO.

In measurement of secondary oxidation products; namely TBA value, the general trend of SHEO antioxidant effect was increasing as the concentration increased and the highest inhibitory effect on formation of secondary products in safflower oil was observed for 4 mg/ml of SHEO.

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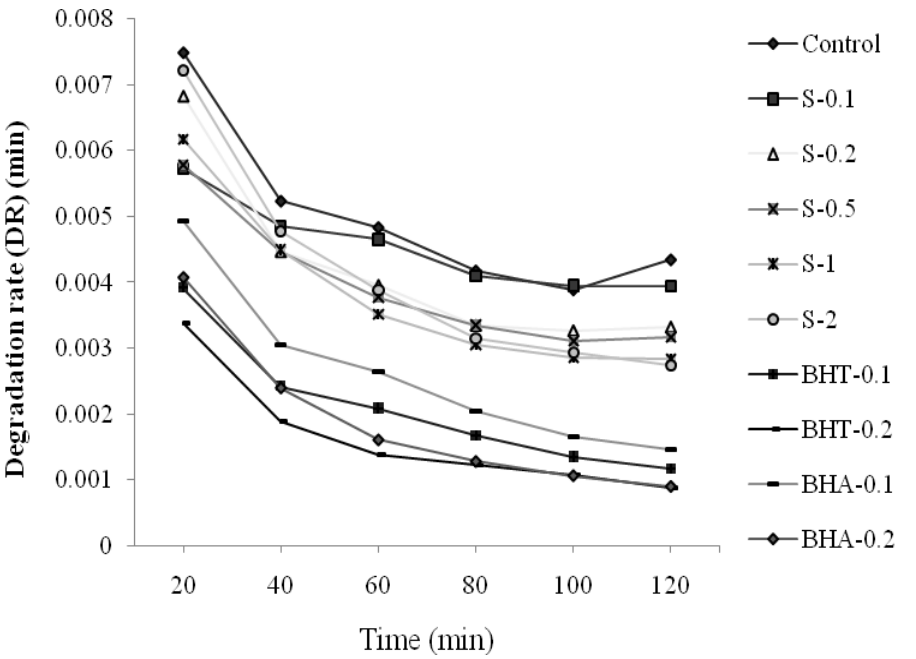


Figure 6- degradation rate (DR) of β-carotene bleaching assay for 0.1-2 mg/ml SHEO

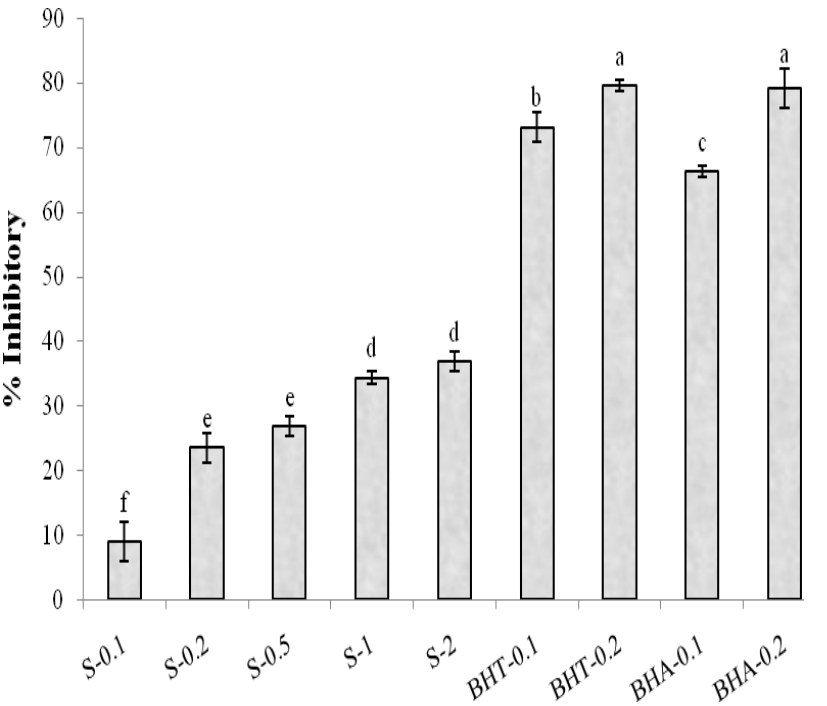


Figure 7- Antioxidant activity of SHEO and BHT and BHA at different concentrations determined by β-carotene bleaching assay

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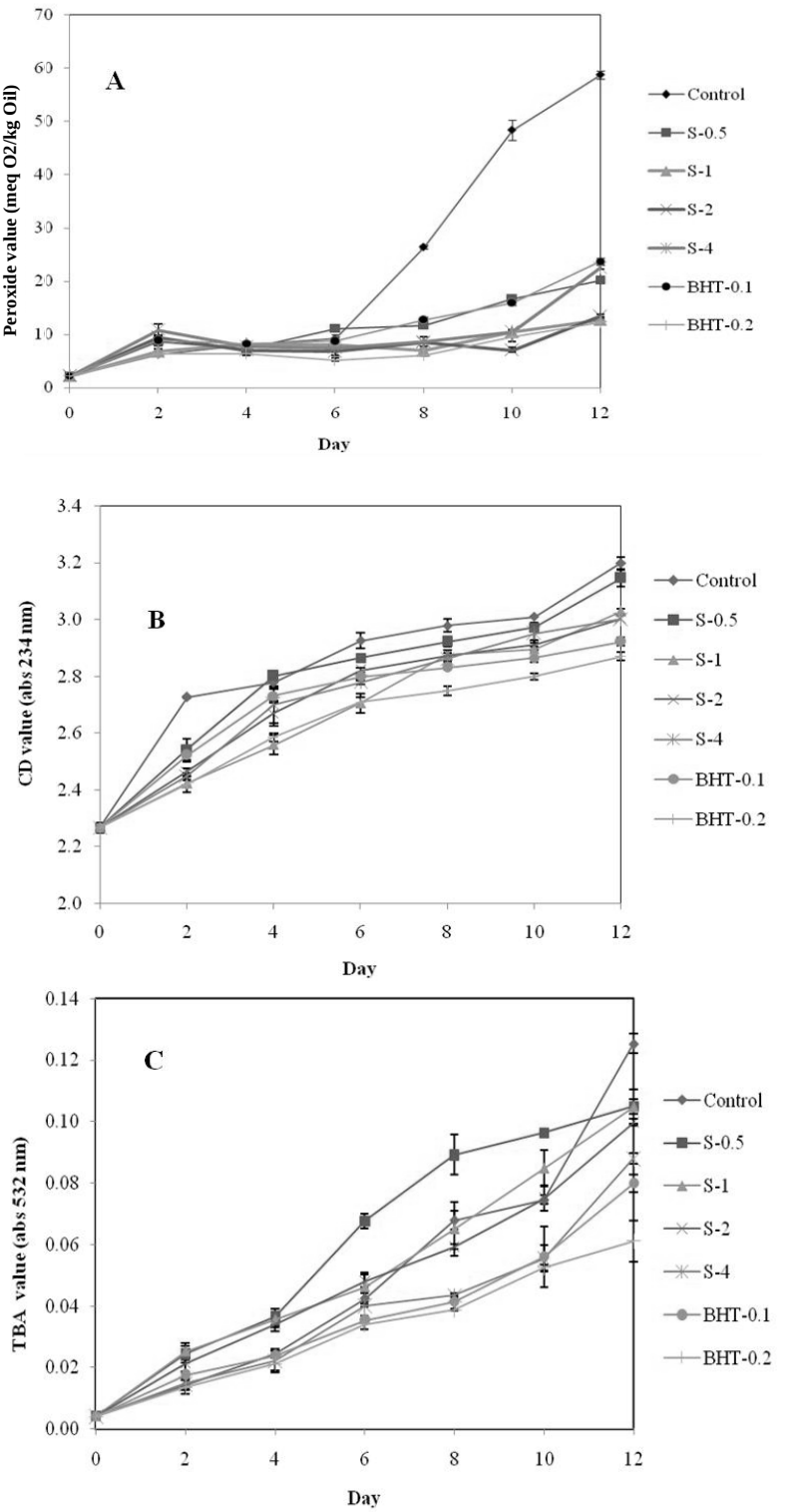


Figure 8 - Effect of SHEO on safflower oil oxidation expressed as peroxide (A), conjugated diens (B) and tiobarbituric acid (C) values during storage at 60 °C

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Discussion

Phenolic compounds are among the secondary metabolites that can be derived from plants in pentose phosphate, shikimate, and phenylpropanoid cycles. Phenolic compounds are found in all parts of plants, and when foods of plant origin are consumed, these phytochemical compounds will act as natural antioxidants in the human, livestock, and poultry diet [20]. Phenols are very important constituents of the plants, and have high scavenging ability of radicals owing to the presence of hydroxyl groups in their structure [21, 22]. According to recent studies, there is a direct relationship between a high total phenol content and antioxidant activity in most spices and plants [1, 23, 24, 25]. Hence, it has been reported that phenol compounds are associated with antioxidant activity and play a major role in preventing fat and oil peroxidation [24]. Plants belonging to the *Laminacea* family with SH have high polyphenolic compounds. In recent studies, polyphenolic compounds have shown good antioxidant activity [22, 27, 28, 30].

Because of being antioxidants, reduction in the absorption of DPPH radical occurred due to the reaction between of the antioxidant and radical molecules. This ultimately ends in scavenging of radicals by hydrogen. That is appeared as change of purple color of the solution into yellow. Thus, usually DPPH$^{\circ}$ has been used as the substrate to evaluate antioxidant activity [22, 27, 28]. It appears that antioxidant effect on scavenging DPPH radical result in its hydrogen donor ability. The DPPH compound is a stable free radical, which requires taking an electron or hydrogen radical to be converted into a stable molecule [22]. The IC_{50} index is concentration of substance that able to have 50% scavenging effect on radical. With increasing the concentration of essential oil, the DPPH radical scavenging activity increased. This is in agreement with the findings of Shahsavari et al. 2008 in DPPH radical assay of the *Bunium persicum* essential oil [29].

In the presence of phenols, ABTS$^{\circ+}$ is partly stable but can react strongly with a hydrogen atom donor such as phenols that formed discolored ABTS. Most phenols and natural products slowly react with the ABTS$^{\circ+}$. So, determination of antioxidant activity depends on time and sample amount; rather than the ABTS$^{\circ+}$ concentration [31]. Adding antioxidants to the radical cation formed (ABTS$^{\circ+}$) reduced it to ABTS and both the intensity and spend time were dependent upon the concentration of antioxidants and reaction time [12].

In the ferric thiocyanate (FTC) method, peroxide formed from the early stages of lipid oxidation is determined, which are primary oxidation products [15, 32, 33, 34]. Peroxide reacts with ferrous chloride and oxidizes ferrous iron to ferric mode, which result in formation of red color thiocyanate complex [34]. As the antioxidant activity increased, concentration of peroxide will be reduced; thus, the intensity of red pigment reduced and absorption is diminished [32]. Lower absorption is represents high antioxidant activity [33]. It is noteworthy that absorption of the control sample in day 10 was less than

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in day 9, owing to conversion of primary lipid oxidation products to secondary products. Lower absorption in the FTC method indicates higher antioxidant activity. This shows that the peroxide amount in early stages of lipid peroxidation is lower than that in the second stage. Furthermore, secondary products are very stable for a period of time [35]. Comparing the antioxidant activity of SHEO with *Centella asiatica* L. extract shows that just SHEO concentration of 8 mg/ml had antioxidant activity similar to that for *Centella asiatica* L. extract, and other SHEO concentrations evaluated had lower antioxidant activity [15].

In the FTC-TBA assay, all SHEO concentrations except 1 mg/ml showed antioxidant activity equal to the *Centella asiatica* L. extract [15].

The β-carotene bleaching method is based on discoloration of yellow color of β-carotene in reaction with radicals formed in oxidation of linoleic acid emulsion. Discoloring rate can be reduced in the presence of antioxidants [38, 39]. Degradation rate of β-carotene is dependent on the extract and essential oil antioxidants. There is a correlation between degradation rate and β-carotene bleaching; such that the extracts and essential oils with lower degradation rate display higher antioxidant activity, and vice versa [16]. Presence of phenolic antioxidants can delayed progress of β-carotene degradation by neutralizing the linoleate free radicals (for example, by applying the oxidation-reduction potential) and other radicals formed in the system. Hence, this mechanism is the basis of determination of antioxidant potential of plant extracts [11, 17].

Some studies have been performed on the effect of antioxidants of plant extracts and essential oils on the stability of oils and foods containing oil. Comparing with the results of other studies for instance on tea seed oil [40], *Zataria multiflora boiss.* [19], and Greek sage (*Salvia fruticosa*) and summer savory (*Satureja hortensis* L.) extracts [26], it was observed that SHEO can appropriately protect safflower oil from oxidation. A study on the antioxidant activity of the *Bunium persicum* essential oil (BPEO) in soybean oil showed that the effect of BPEO concentration of 0.06% was comparable to that of 0.02% BHA (Shahsavari *et al*., 2008). Goli *et al.* found that green skin pistachio extract in 600 ppm is comparable with 200 ppm of synthetic antioxidant, BHT and BHA, in delaying oxidation in soybean oil [41]. Yasoubi *et al.* compared the antioxidant activity of pomegranate skin extract in soybean oil with BHA and BHT and report that pomegranate skin extract with the concentration of 0.05% has higher antioxidant effect than 0.02% BHA and BHT [42].

Conclusion

Considering the results of previous research and the current study, it can be concluded that SHEO has antioxidant effects in safflower oil. It is hoped that common synthetic antioxidants that have harmful effects on human health be replaced by such natural materials with useful properties.

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