Shanasayi Tarkiabat va Barsee Fualiyat Ayni Aeksidayi Aassasi Do Jumeeet Nuroozk Iran (Tere Tuglan)

Keywords. BCB, DPPH, sage, TBARS, volatile oil

Abstract. Salvia leiriifolia Benth. from Lamiaceae family is a plant native to Iran and Afghanistan with significant applications in medical, pharmaceutical and food industries. The aim of current investigation was to evaluate the composition and antioxidant activity of essential oils (EOs) of S. leiriifolia growing naturally in Neyshabur and Bajestan (Northeast of Iran). The aerial parts of the plant were subjected to hydro-distillation and the EOs were analyzed with GC/MS. According to the results, 1,8-cineole (20.24%, 26.39%), α-pinene (15.14%, 14.39%) and β-pinene (24.33%, 26.01%) were the main constituents of the EOs of the plant populations in Neyshabur and Bajestan, respectively. Antioxidant activity of the EOs was measured by three different methods, 2,2-diphenyl-1-picrylhydrazyl (DPPH), thiobarbituric acid reactive species (TBARS) and β-carotene bleaching (BCB). The total EO and three standards (α-pinene, β-pinene and 1,8-cineole) displayed a significant antioxidant effect in all assays. Antioxidant activities obtained by means of TBARS and BCB methods were higher than those measured by the DPPH assay. Moreover, BCB was proved to be the most appropriate method for measurement of antioxidant activity. The EOs of the plants grown in Neyshabur showed stronger antioxidant effects in comparison with those grown in Bajestan.

Keywords. BCB, DPPH, sage, TBARS, volatile oil
INTRODUCTION

Oxidation of biomolecules with free radicals could lead to cell injury and death (Wang et al., 2007). Reactive oxygen species oxidize lipids containing polyunsaturated fatty acids readily. Coronary heart disease, aging, stroke, Parkinson, multiple sclerosis, carcinogenesis and Alzheimer are results of lipid peroxidation. An increasing investigations have been done to find anti-oxidative drugs which participate as radical scavengers in living organisms (Emami et al., 2007). Widely-used synthetic antioxidants in food products have been known to cause negative health effects. Studies on natural compounds as potential antioxidants have been of great interests for specialists (Fasseasas et al., 2007). Fruits, vegetables, nuts and whole grains have been studied in recent years as sources of natural plant antioxidants (Kulisic et al., 2004; Wang et al., 2007; Bohn et al., 2010). Essential oils (EOs) from various aromatic plants have been identified as strong natural antioxidants and a lot of studies on their antioxidant properties have been repeate- dly reported so far (Ruberto & Baratta, 2000; Kulisic et al., 2004; Kelen & Tepe, 2008; Okoh et al., 2011; Rowshan & Bejeli, 2013).

The EOs of some genera of the Lamiaceae family are potential candidates for exhibiting antioxidant and radical-scavenging activities (Emami et al., 2007). Numerous species of Salvia L. have been used in folk medicine for their wide variety of pharmacological properties. They were subjected to extensive investigations for identification of the biologically active compound (Bozan et al., 2002).

The S. officinalis L. EOs exhibited remarkable antioxidant activity (IC50 = 7.70 ± 0.90 μg/ml) (Bouaziz et al., 2009). The EO of S. tomentosa Miller was particularly found to possess strong antioxidant activity (Tepe et al., 2005). EOs of three different Salvia species (S. aucheri Benth. var. aucheri, S. aramiensis Rech.f and S. pilifera Montbret & Aucher) were screened for their possible antioxidant activity. Antioxidant activity of S. aramiensis was found to be higher than others (Kelen & Tepe, 2008).

Salvia leriifolia Benth. is a perennial herbaceous plant which is native to Razavi Khorassan and Semnan provinces of Iran. This plant has different vernacular names such as Norouzak and Jobleh. Salvia leriifolia has different pharmacological activities such as anticonvulsant (Hosseinzadeh & Arabsanavi, 2001), anti-ischemia (Hosseinzadeh et al., 2007), anti-inflammatory (Hosseinzadeh & Yavary, 1999; Hosseinzadeh et al., 2003) and antinoceptive (Hosseinzadeh et al., 2003), antioxidant (Farhoosh et al., 2004), antibacterial (Habibi et al., 2000) and antiulcer effects (Hosseinzadeh et al., 2000). Little information is available on the antioxidative nature of its EO for treatment of Alzheimer and acetyl-cholinesterase inhibitory. This plant has been introduced as an herbal medicine in toxicological and clinical trial evaluations (Hosseinzadeh et al., 2009; Savelev et al., 2004; Loizzo et al., 2009). An attempt was made in this study to identify the composition and to examine the antioxidant activity of S. leriifolia EOs in its flowering stage. In order to overcome possible methodology limitations, three different assay methods were employed, i.e. 2, 2’-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging me-thod, the thiobarbituric acid reactive species (TBARS) assay and the β-carotene bleaching (BCB) test.

MATERIAL AND METHODS

Mass spectrophotometric (GC-MS) analysis

Plant material

Salvia leriifolia leaves were collected from the Bajestan and Neyshabur regions, Northeast of Iran, during the flowering stage. The specimen was identified by Mohammadreza Joharchi (FU-MH) and deposited by the voucher number of A.A.Basiri 12835. The dried leaves were powdered. Three analytical standards (α-pinene, β-pinene and 1,8-cineole) were purchased from Sigma-Aldrich, USA.

Isolation of the essential oils

The powdered aerial parts of S. leriifolia L. (300 g) were subjected to hydro-distillation using a Clive-genger-type apparatus for 3 hours. After dehydration by means of anhydrous sodium sulfate, the slightly yellow-colored oil was obtained and stored at -20°C prior to GC/MS and antioxidant analysis.

Gas chromatography-mass spectrometry

The GC-MS analyses were performed using an Agilent 5975 apparatus with HP-5ms column, interfaced with a quadruple mass detector and a computer equipped with Wiley 7 n.l library.

The constituents of the oil were identified by calculation of their retention indices under programmed temperature conditions for n-alkanes (C8-C20) and the oil on a CP-Sil 8CB column. The individual compounds were identified by comparing their mass spectra and retention indices (RI) with those of authentic samples and those being given in literatures (Adams, 2007).
Determination of antioxidant activity by means of the DPPH radical scavenging method

Hydrogen atoms or electrons donation ability of the corresponding oils was measured by the bleaching of purple colored methanol solution of DPPH. Fifty microliter of various concentrations (0.1, 0.5, 1, 2, 4, 8, 16 μl/ml) of the EOs and three main components of the EO, as well as quercetin and ascorbic acid in methanol, was added to 2.5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, absorbance was read against a blank at 517 nm. Inhibition free radical DPPH in percent (I%) was calculated in following way:

\[
I\% = \left(1 - \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}\right) \times 100
\]

Determination of antioxidant activity with TBARS assay

A modified TBARS assay was used to measure the potential antioxidant capacity using homogenized egg yolk as lipid rich media. 0.5 ml of 10% (w/v) tissue homogenate and 0.1 ml of sample solutions to be tested in methanol. 0.05 ml 2,2’-azobis(2-amidinopropane) dihydrochloride solution (0.07 M) in water was added to induce lipid peroxidation. 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml 0.8% (w/v) sodium dodecyl sulfate solution was added and the resulting mixture vortexed, and then heated at 95°C for 60 min. After cooling, 5.0 ml of butanol was added to each tube, then extensively vortexed and centrifuged at 1200g for 10 min. The experiment was carried in triplicate. The absorbance of the organic upper layer was measured using a Shimadzu UV-3100 scanning spectrophotometer, set at 532 nm. All the values were based on the percentage antioxidant index (AI%):

\[
AI\% = \left(1 - \frac{T}{C}\right) \times 100
\]

Where C is the absorbance value of the fully oxidized control and T is the absorbance of the test sample. Vitamin E and butylated hydroxytoluene (BHT) were used as positive controls (Kulisic et al., 2004).

Determination of antioxidant activity with BCB test

Antioxidant activity of the S. leriifolia EO compounds was determined according to a slightly modified version of the β-carotene bleaching method. 0.5 mg of β-carotene in 1 ml of chloroform was added to 25 μl of linoleic acid and 200 mg of Tween 40 emulsifier mixture. After the evaporation of chloroform under vacuum, 50 ml of oxygenated distilled water was added and the mixture was sonicated with RPMI-1640 for 1 minute. Five milliliter of this mixture were transferred into different test tubes (200 μl) containing different concentrations of the sample (concentrations of stock solutions were 0.1, 0.5, 1.0, 2.0, 3.0 and 4.0 g/l). As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. The emulsion system was incubated for 2 h at 50°C and measured six time interval at 4 g/l. All determinations were performed in triplicate. The percentage inhibition was calculated from the data with the slightly modified formula:

\[
\%\text{inhibition} = \left[\frac{(A_A(120) - A_C(120))}{(A_C(0) - A_C(120))}\right] \times 100
\]

Where AA (120) is the absorbance of the antioxidant at t=120 min, AC (120) is the absorbance of the control at t=120 min, and AC (0) is the absorbance of the control at t=0 min. BHT was used as positive control (Ozkan & Erdogan, 2011).

Statistical analysis

The data were analyzed statistically using INSTAT 3.0 software. The significant level was ascertained by one way analysis of variance (ANOVA), followed by Tukey multiple comparison test. Results were experienced as means ± SD and P values of <0.001 were considered as significant. Graphs were drawn by Graph Pad Prism3.0 and Microsoft Excel.

RESULTS AND DISCUSSION

Chemical composition of S. leriifolia essential oil

The dried S. leriifolia leaves yielded 0.6% (v/w) of EO. The constituents of the EO’s were listed in Table 1. Thirfty nine compounds (96.95% of the EO), and thirthy five compounds (93.77% of the EO) were identified in the EO of S. leriifolia collected from Neyshabur and Bajestan. The fundamental compounds in the EO’s were monoterpenic hydrocarbons. The major constituent of Neyshabur and Bajestan S. leriifolia oils were α-pinene (14.39, 15.14%), β-pinene (26.01, 24.33%) and 1,8-cineol (eucalyptol) (26.39, 20.24%), respectively. These EO’s contained (77.17, 70.66%) monoterpenes and (19.78, 23.11%) sesquiterpenes, respectively.
It is certainly true that there are little differences in the chemical composition and main component of *S. leriifolia* EO among two localities. The different climatic conditions have a negligible impact on the chemical composition of the EO’s. However, a number of compounds like nopinone, verbena- one, β-copaene, α-calacorene and neryl isovalerate were not identified in Bajestan EO (Table 1). Hosseinzadeh et al., (2009) noted that β-pinene (31.5%), 1, 8 cineole (24.7%) and α-pinene (17.5%) were the main components in the EO’s of *S. leriifolia* plants grown in the southern regions of Khorassan and Semnan provinces.

Their finding is extremely similar to the results of the present study. In addition, Monfared and Ghorbanli (2009) recorded 1,8-cineole (20.04%), camphor (18.84%), α-pinene (16.49%) and camphene (10.94%) as the main constituents in the EO of plants grown in Bardaskan, Kashmar, Iran (Monfared & Ghorbanli, 2009). According to another study carried out by Loizzo et al. (2009), camphor (10.5%), 1, 8-cineole (8.6%), camphene (6.2%) and α-pinene (4.7%) were the main components of *S. leriifolia* EO from the southern regions of Khorassan (Loizzo et al., 2009).

These findings are not in agreement with the results presented here. The changes in the EO compositions might be the consequence of several different aspects including climatic, seasonal, geographical, and geological conditions (Perry et al., 1999).

**Antioxidant activity**

**DPPH radical scavenging method**

In the DPPH assay, the values of 17.8% and 37.2% were determined as free radical scavenging activity of *S. leriifolia* EO in Bajestan and Neyshabur, respectively (Fig.1), whereas the values of 3.1%, 11.6% and 2% were estimated for 1, 8-cineole, α-pinene, β-pinene, respectively (in 16 μl/ml) (Fig. 2). The samples were less effective in comparison with ascorbic acid (95.2%) and quercetin (94.2%) as synthetic antioxidant agents. Higher antioxidant activity of *S. leriifolia* EO collected from Neyshabur (31.6%) than EO of plants grown in Bajestan (24.1%) might be partially due to the more amounts of oxygenated monoterpenes, which are strong antioxidant compounds (Ruberto & Baratta, 2000).

**Thiobarbituric acid reactive species assay**

As shown in Figures 3 and 4, at the concentration of 40 mg/ml in TBARS test, antioxidant activity of *S. leriifolia* oil from Bajestan was 59.2%. It was 57.8% for plants grown in Neyshabur. The values of 35.5%, 17.2% and 35.5% were measured as antioxidant indices for 1, 8-cineol, α-pinene and β-pinene, respectively. EOs exhibited almost the same antioxidant index. All the samples showed that less antioxidant activity as compared to vitamin E (91.5%) and BHT (71.24%) with the same concentration.

**β-carotene bleaching method**

*Salvia leriifolia* EO from Bajestan (48.7%) and Neyshabur (52.7%) and their major components including 1,8-cineole (32.2%), α-pinene (36.9%), β-pinene (38.8%) bleached β-carotene at the concentration of 4 mg/ml (Figs. 5 and 6) in BCB. Absorbance of β-carotene in the presence of total EO, as well as its constituents and positive control (sample with no antioxidant), showed a gradual decrease (Fig. 7).

A descending order in bleaching rate can be demonstrated as follows: 1,8-cineole >β-pinene >α-pinene>EO of plants harvested from Bajestan>EO of plants grown in Neyshabur > BHT. *Salvia* species have been known as potent natural antioxidants (Rowshan & Bejeli, 2013). Antioxidant activities for the extracts of various *Salvia* species have been described so far. (Tepe et al., 2004, 2005).

Moreover, the anti-oxidant activities of EOs belonging to different *Salvia* species such as *S. officinalis* (IC₉₀ values 22 mg/ml), *S. aramienesis* (IC₉₀ values 12.26 mg/ml), *S. aucharı* (IC₉₀ values 0.018 mg/ml) and *S. pilifera* (IC₉₀ values 0.024 mg/ml) have been demonstrated by DPPH assay and for *S. microphylla* (IC₉₀ values 0.77 mg/ml) have been measured by BCB test (Lima et al., 2012; Bouajaj et al., 2013; Kose et al., 2013). The EO of *S. eremophila* was almost inactive in DPPH and acting weakly in BCB test (Ebrahimabadi et al., 2010).

A number of studies on the antioxidant activity of *S. leriifolia* extract have been carried out (Farhoosh et al., 2004; Hosseinzadeh et al., 2009; Loizzo et al., 2010). However, there is only one study on the antioxidant activity of its EO.

According to a study executed by Loizzo et al. (2009), *S. leriifolia* oil exhibited a promising antioxidant activity by DPPH assay with an IC₉₀ 2.26 μl/ml.
Table 1. Chemical composition (%) of the essential oil of *S. leriifolia* leaves, collected from Neyshabur & Bajestan

<table>
<thead>
<tr>
<th>Compound</th>
<th>(^{a}RI)</th>
<th>(^{b}RI)</th>
<th>EO (N) %</th>
<th>EO (B) %</th>
</tr>
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<tbody>
<tr>
<td>(\alpha)-Thujene</td>
<td>932</td>
<td>930</td>
<td>0.65</td>
<td>0.75</td>
</tr>
<tr>
<td>(\alpha)-Pinene</td>
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<td>Camphene</td>
<td>957</td>
<td>954</td>
<td>1.46</td>
<td>3.3</td>
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<td>Sabinene</td>
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<td>975</td>
<td>0.38</td>
<td>0.79</td>
</tr>
<tr>
<td>(\beta)-Pinene</td>
<td>987</td>
<td>979</td>
<td>26.01</td>
<td>24.33</td>
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<tr>
<td>(\delta)-3-Carene</td>
<td>1015</td>
<td>1011</td>
<td>1.1</td>
<td>1.25</td>
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<tr>
<td>1,8-Cineole</td>
<td>1039</td>
<td>1031</td>
<td>26.39</td>
<td>20.24</td>
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<tr>
<td>(\gamma)-Terpinene</td>
<td>1062</td>
<td>1059</td>
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<td>0.83</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>1091</td>
<td>1088</td>
<td>0.48</td>
<td>0.42</td>
</tr>
<tr>
<td>Linalool</td>
<td>1098</td>
<td>1090</td>
<td>0.1</td>
<td>0.12</td>
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<tr>
<td>Nopinone</td>
<td>1142</td>
<td>1140</td>
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<td>—</td>
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<tr>
<td>Camphor</td>
<td>1150</td>
<td>1146</td>
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<tr>
<td>Pinocarvone</td>
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<td>1164</td>
<td>0.7</td>
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<td>Terpinen-4-ol</td>
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<td>1177</td>
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<td>Myrtenol</td>
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<td>1195</td>
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<td>0.19</td>
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<td>1513</td>
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<tr>
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<td>1674</td>
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<table>
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<th>Grouped compounds</th>
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<td>Monoterpane hydrocarbons</td>
<td>45.69</td>
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<td>Oxygenated monoterpenes</td>
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<td>Sesquiterpene hydrocarbons</td>
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<td>6.93</td>
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<tr>
<td>Total</td>
<td>96.95</td>
<td>93.77</td>
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\(^{a}RI\): The retention index calculated from retention times relative to C8-C20 n-alkanes on a CP-Sil 8 CB column.

\(^{b}RI\): The retention index from reference data (Adams, 2007).

N: Neyshabur; B: Bajestan
**Fig. 1.** Free radical-scavenging activity of *S. leriifolia* essential oil collected from Neyshabur (N) and Bajestan (B) in DPPH assay.

**Fig. 2.** Free radical-scavenging activity of α-pinene, β-pinene and 1,8-cineole in DPPH assay.

**Fig. 3.** Antioxidant activity of the *S. leriifolia* essential oil collected from Neyshabur (N) and Bajestan (B) in TBARS test.
Fig. 4. Antioxidant activity of the α-pinene, β-pinene and 1,8-cineole in TBARS test.

Fig. 5. Antioxidant activity of *S. leriifolia* essential oil collected from Neyshabur (N) and Bajestan (B) in BCB test.

Fig. 6. Antioxidant activity of α-pinene, β-pinene and 1,8-cineole in BCB test.
On the contrary, the EO of *S. leriifolia* was almost inactive in DPPH test of the current study and an inhibition percentage of less than 38% was recorded for the oil concentrations up to 16 μl/ml. EO’s generally have low solubility in DPPH method; therefore, the DPPH test could not be reliable for the measurement of the antioxidant activities of such materials (Lima *et al*., 2012). In this survey, however, the EO’s from *S. leriifolia* in Bajestan and Neyshabur had IC50 values of 5.7 and 2.7 mg/ml in BCB test and 18.9 and 15.2 mg/ml in TBARS assay. This study and the aforementioned ones confirm the presence of moderate to good antioxidant potentials for the EO’s of the *Salvia*.

The EO of *S. leriifolia* contains some active components such as 1,8-cineole, α-pinene and β-pinene, which have been reported to exhibit an antioxidant activity. In general, *S. leriifolia* EO showed higher activity than its components in the three systems. It is very difficult to ascribe the antioxidant power of a total EO to one or some active fractions, which could be due to the fact that an EO always contains a mixture of different chemical components. Not only major but minor compounds also may make significant contributions to the oil activity (Wang *et al*., 2007).

α-Pinene and β-pinene are in monoterpenic hydrocarbons classes. Presence of strongly activated methylene groups in these molecules probably accounts for their antioxidant behavior (Giweli *et al*., 2012). However, the relative high activity of the aforesaid monoterpenes is also confirmed by the TBARS assay. 1, 8-cineole is classified as an-oxygenated monoterpane. Many examples of different functional compounds (alcohols, aldehydes, ketones, ethers, etc.) belong to this group. The antioxidant activity of these compounds depends on the presence of particular molecular moieties. Alcohols are the most active materials. Ethers (such as 1,8-cineole, the monoterpenic cyclic ether) has less antioxidant activity (Ruberto & Baratta, 2000).

Since the specificity and sensitivity are different for each method used, application of an analytical eclectic method is ideal to evaluate the effectiveness of antioxidants accurately (Kelen & Tepe, 2008). This study also suggested that a single assay might not be sufficient to estimate the antioxidant activity of a plant extract or an EO sample. The co-application of three methods was turned out to be a good technique for evaluation of the antioxidant activity of *S. leriifolia* EO and could be recommended for similar investigations. In addition, the multi-concentration measurements provide a more comprehensive picture of a plant EO antioxidant activity in general.

In conclusion, the measured antioxidant power depends on the method employed and the concentration, intrinsic nature and physico-chemical properties of the materials studied (Kulisic *et al*., 2004; Ruberto & Baratta, 2000).

It was also confirmed that the exhibition of antioxidant activities of a single EO specimen may differ according to its concentration and the type of antioxidant assay (Kelen & Tepe, 2008).

DPPH and TBARS similarly allow testing of both hydrophilic and lipophilic substances (Magalhaes *et al*., 2008).
Moreover, both model systems, BCB and TB-ARS, should be considered important since they allow us to follow the primary or secondary steps of the oxidative process.

It is necessary to assess antioxidant effectiveness in model systems dealing with the primary and secondary steps of lipid oxidation especially when food quality is under investigation (Ruberto & Baratta, 2000).

CONCLUSION

From the results above we could infer that the antioxidant activity of *S. leriifolia* EO is the cooperating result of its composition. In the extracted EO’s, oxygenated monoterpenes and monoterpenic hydrocarbons are mainly responsible for its antioxidant potential. The oxygenated monoterpenes were found out to be the main components of *S. leriifolia* EO. The result could be of interest to those in charge of food industries in finding the possible alternatives to synthetic preservatives. In this context, *S. leriifolia* EO showed interesting results, being one of the best functioning antioxidants in terms of neutralizing the free radicals.

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