LC-MS/MS Analysis of Phenolic Compounds and In Vitro Antioxidant potential of *Stachys lavandulifolia* Vahl. var. *brachydon* Boiss.

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**Abstract.** The identification and quantification of phenolic compounds of *Stachys lavandulifolia* Vahl. var. *brachydon* Boiss. by LC-MS/MS (Liquid Chromatography-tandem Mass Spectrometry) technique is the main purpose of the current study. The high concentrations of quinic acid (2534±12 ppb) and chlorogenic acid (1882±92 ppb) were detected by LC-MS/MS. Another goal of the study is to evaluate the antioxidant activities of both ethanol and aqueous extracts of the plant material. The antioxidant potentials of extracts were determined by using five different *in vitro* methods including; ABTS (2,2′-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid), DPPH (1,1-diphenyl-2-picryl-hydrazyl), FRAP (Ferric ions Reducing Antioxidant Power), CUPRAC (Cupric ions Reducing Antioxidant Power), and ferric thiocyanate methods. The results revealed that the aqueous and ethanol extracts of *S. lavandulifolia* leaves have good antioxidant potential with high phenolic content.

**Introduction**

The *Stachys* is the largest genera of the Lamiaceae with its nearly 300 species. They mostly exist in the warm regions such as Mediterranean, South-West Asia, America and Southern Africa [1]. In the Flora of Turkey, *Stachys* is represented by 86 species (109 taxa), 37 of which are endemic and divided into fourteen sections [2]. *Stachys lavandulifolia* has three varietes (brachyodon, glabrescens, and lavandulifolia). *Stachys lavandulifolia* Vahl. var. *brachydon* Boiss. separated from other varietes by different structure of leaves and lenght of calyxes [3].

Some previous phytochemical identification studies of *Stachys* species were demonstrated the phenolic and flavonoid glycosides [4], terpenoids and steroids [5], flavonoid constituents [6]. Also, phytochemical investigations on different *Stachys* taxa have shown that they have various pharmacological effects, such as anti-inflammatory, antitoxic, hypoazotemic, antihepatitis, antibacterial and antioxidant [7, 8]. Besides, some *Stachys* taxa have been reported in folk medicine to treat genital tumors, sclerosis, inflammatory tumors, cancer, and ulcers. Furthermore, some of them are used in phyotherapy, possessing sedative, antispasmodic, diuretic and emmenagogue activities as a tea preparations [9]. *Stachys* genera is classified in Lamiaceae family which has been reported widely for their phenolic and flavonoid contents as well as some biological properties [10-13]. The major compounds of *S. lavandulifolia* were determined by some previous reports [14, 15].

Best of our knowledge there is no comprehensive research on the literature about phenolic content and antioxidant activity of *Stachys lavandulifolia* Vahl. var. *brachydon* Boiss.

We investigated antioxidant potential of *Stachys lavandulifolia* Vahl. var. *brachydon* Boiss. (*S. lavandulifolia*) by five *in vitro* common methods; ABTS, DPPH, CUPRAC (Cupric ions Reducing Antioxidant Power), FRAP, and ferric thiocyanate techniques, separately. According to the data that obtained from the mentioned methods, *S. lavandulifolia* extracts showed a great deal of antioxidant activity on scavenging of radicals and reducing ions properties. Additionally, the data of LC-MS/MS method displayed the organic compounds of *S. lavandulifolia*. Quinic acid and chlorogenic acid were the most abundant phenolic compounds with respect to the results.
Theory

Antioxidant potential is mostly related with phenolic content. Owing to the high biological activity and phenolic contents, detailed further studies about of *S. lavandulifolia* might be beneficial on food industry and pharmacology.

Materials and Methods

The plant material

*Stachys lavandulifolia* Vahl var. brachydon Boiss. leaves were collected from Bingol, a South Eastern city of Turkey, on July 2016. The samples were identified by Dr. Ömer Kılıç based on the Flora of Turkey and East Aegean Islands, volume VII [3].

Chemicals

The chemicals that used for antioxidant assays as well as standard organic compounds that used for LC-MS/MS analyses were purchased from Fluka (Germany) and Sigma-Aldrich (Germany) companies.

Preparation of aqueous extract

The air dried leaves of *S. lavandulifolia* (20 g) were powdered and mixed with 200 mL distilled water (w/v:1/10). The mixture was homogenized by a magnetic mixer about 12 h, at room conditions. The mixture was filtered with filter paper. The elution was frozen and lyophilized in a lyophilizer (Labconco, Freezone 1L) at 5 mm Hg pressure and at -50 °C. Then, the lyophilized sample was keep in reserve at -30 °C until used.

Preparation of ethanol extract

The air dried leaves of *S. lavandulifolia* (20 g) were powdered and mixed with 200 mL ethanol (w/v:1/10). The mixture was homogenized by a magnetic mixer about 12 h, at room conditions. The mixture was filtered with filter paper. The elution was evaporated at 50 °C with a rotary evaporator (Heidolph 94200, Bioblock Scientific) and sample was keep in reserve at -30 °C until used.

LC-MS/MS studies for phenolic content

LC-MS/MS technique was used for identification of phenolic compounds quantitatively. Phenolic content of *S. lavandulifolia* was compared to standard mix of twenty-seven organic compounds.

The diluted ethanol extract (1 mg/mL) was used for LC-MS/MS analyses. LC-MS triple quadrupole mass spectrometer (Shimadzu, 8040) was the main part of the LC-MS/MS system. The ionizations were detected by ESI. The binary pumps (LC-30AD), column oven (CTO-10ASvp) degasser (DGU-20A3R), and auto sampler (SIL-30AC) were integrated to the LC system. The chromatographic separation was performed on a C18 (150 mm × 4.6 mm, 3 μm) reversed phase analytical column (Inertsil ODS-4). The mobile phase A consisted of 5 mM ammonium formate, water and 0.1 % formic acid. The mobile phase B consisted of 5 mM ammonium formate, 0.1 % formic acid and methanol. The injection volume of sample was 4 μL. HPLC was runned at 0.5 mL/min flow. The multiple reaction monitoring (MRM) mode was used to quantify the analyzes. The optimum ESI conditions were set as 350°C for interface temperature, 250°C for DL temperature, and 400°C for heat block temperature, 3 L/min for nebulizing gas flow and 15 L/min for drying gas flow. The analyses of samples were carried out after three transitions for the sample. First transition was for quantitative aim and second and third transitions were for verification [16].
**In vitro antioxidant activity assays**

**Ferric thiocyanate method**

The ferric thiocyanate method was used to evaluate the effects of WS (aqueous extract of *S. lavandulifolia*) and ES (ethanol extract of *S. lavandulifolia*) on the prevention of peroxidation of linoleic acid emulsion [17]. The samples of WS and ES (20 μg/mL) were prepared by diluting in 2.5 mL of sodium phosphate buffer (0.04 M, pH 7.0) and these were added to 2.5 mL of linoleic acid emulsion in sodium phosphate buffer (0.04 M, pH 7.0). The linoleic acid emulsion was prepared by homogenizing 15.5 μL of linoleic acid, 17.5 mg of Tween-20 as emulsifier, and 5 mL phosphate buffer (pH 7.0). The control without sample was composed of 2.5 mL of linoleic acid emulsion and 2.5 mL 0.04 M sodium phosphate buffer (pH 7.0). The reaction mixtures (5 mL) were incubated at 37 °C. The peroxide levels were determined by reading the absorbance at 500 nm in a spectrophotometer (Shimadzu, UV-1208 UV–VIS Spectrophotometer, Japan) after reactions with FeCl₂ and thiocyanate at intervals during incubation. The peroxides formed during linoleic acid peroxidation oxidize Fe²⁺ to Fe³⁺, and Fe³⁺ forms a complex with thiocyanate that has a maximum absorbance at 500 nm. The assay steps were repeated every 10 h until the maximum was reached. The percentage of inhibition was calculated at this point (100 h). The linoleic acid mixture without the addition of sample was used as control.

**FRAP reducing method**

Transforming ferric ions to ferrous ones indicate antioxidant reducing potential of a substance. Antioxidants do this like a proton donor or single electron transfer. The extracts (WS and ES) and standard antioxidants were prepared in different concentrations (10–30 μg/mL), respectively. The mixtures were incubated for 20 min (50 °C) after adding 1 mL potassium ferricyanide (1 %) and 1 mL buffer solution (sodium phosphate 0.2 M; pH 6.6). One milliliter TCA solution (10 %) was added to complete the reaction. Finally, 0.25 mL FeCl₃ (0.1 %) was put in test tubes. The absorbances at 700 nm were reported by using a UV spectrophotometer [18].

**CUPRAC reducing method**

Transforming cupric ions (Cu²⁺) to cuprous ones (Cu⁺) mostly related with antioxidant reducing potential of a substance. In this technique, 0.5 mL neocuproine (7.5×10⁻³ M), 0.5 mL CuCl₂ (0.01 M), and 0.5 mL buffer solution (acetate, 1 M: pH 5.0) were added to each tubes, respectively. Then, different concentrations (10–30 μg/mL) of extracts were added. The volumes were completed to 4 mL with purified water. The samples were waited at room conditions for 30 min and their absorbances at 450 nm were reported. Increasing absorbance indicates reducing capacity of a sample [19].

**ABTS radical scavenging method**

The ABTS⁺⁺ radical scavenging activity of *S. lavandulifolia* extracts (WS, ES) were evaluated according to the method that previously reported [20]. ABTS⁺⁺ is blue–green in colour with a characteristic absorbance at 734 nm. ABTS⁺⁺ cation radical was produced by reacting ABTS (2mM) in H₂O and potassium persulphate (2.45 mM) at room temperature for 12 h. The ABTS⁺⁺ solution was diluted with phosphate buffer (0.1 M, pH 7.4) to achieve an absorbance of 0.750±0.025 at 734 nm. Then, 1 mL of ABTS⁺⁺ solution was added to 3 mL of extract solution in methanol at different concentrations (10–30 μg/mL) of the samples and standards. These samples were vortexed and incubated in the dark for 30 min. Later, absorbances at 734 nm were measured relative to a blank. Decreased absorbance of the samples indicates ABTS⁺⁺ cation radical scavenging activity.

**DPPH radical scavenging method**

The DPPH radical scavenging activity of *S. lavandulifolia* extracts (WS, ES) were evaluated according to the method that previously reported [21]. The DPPH method is based on the reducing absorbance of DPPH radicalic solution because of radical scavenging effects of antioxidants. For this purpose, different concentrations (10–30 μg/mL) of samples and standard compounds were...
prepared in 2 mL ethanol. Then, 1 mL DPPH radical solution (0.1 mM) was added to each sample and left in the dark for 30 min. The absorbances were measured at 517 nm using a spectrophotometer.

Results and Discussion

Phenolic compounds contain hydroxyl group on their chemical structure. Phenolic compounds exist on natural plants are showing an extensive range of physiological feature. Also, they have many biological activities such as antioxidants and antiradical [22]. In this study, phenolic content of *S. lavandulifolia* was determined by UHPLC-ESI-MS/MS method. The most abundant organic compounds were detected as quinic acid (2534±12 ppb) and chlorogenic acid (1882±92 ppb) among the twenty-seven standard compounds. Moreover, lower amounts of another compounds were identified and were given on Table 1. Also, the chromatograms of standards (Fig. 1) and *S. lavandulifolia* were presented (Fig. 2).

Table 1. Organic compounds of *S. lavandulifolia* and LC-MS/MS parameters.

<table>
<thead>
<tr>
<th>No</th>
<th>Analytes</th>
<th><em>RT</em></th>
<th>*Parent ion (m/z)</th>
<th>Ionization Mode</th>
<th><em>R</em></th>
<th><em>RSD%</em></th>
<th>Linearity Range (µg/L)</th>
<th>*LOD/LOQ (µg/L)</th>
<th>Recovery (%)</th>
<th><em>U</em></th>
<th><em>Amount</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Quinic acid</td>
<td>3.32</td>
<td>190.95</td>
<td>Neg</td>
<td>0.9927</td>
<td>0.0388</td>
<td>250-1000</td>
<td>22.3 / 74.5</td>
<td>103.3</td>
<td>4.8</td>
<td>2534±122</td>
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<td>2</td>
<td>Malic acid</td>
<td>3.54</td>
<td>133.05</td>
<td>Neg</td>
<td>0.9975</td>
<td>0.1214</td>
<td>250-1000</td>
<td>19.2 / 64.1</td>
<td>101.4</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>tr-Aconitic acid</td>
<td>4.13</td>
<td>172.85</td>
<td>Neg</td>
<td>0.9933</td>
<td>0.3908</td>
<td>250-1000</td>
<td>15.6 / 51.9</td>
<td>102.8</td>
<td>4.9</td>
<td></td>
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<tr>
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<td>Gallic acid</td>
<td>4.29</td>
<td>169.05</td>
<td>Neg</td>
<td>0.9901</td>
<td>0.4734</td>
<td>25-1000</td>
<td>4.8 / 15.9</td>
<td>102.3</td>
<td>5.1</td>
<td></td>
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<td>Chlorogenic acid</td>
<td>5.43</td>
<td>353</td>
<td>Neg</td>
<td>0.9932</td>
<td>0.1882</td>
<td>250-1000</td>
<td>7.3 / 24.5</td>
<td>99.7</td>
<td>4.9</td>
<td>1882±92</td>
</tr>
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<td>Protocatechuic acid</td>
<td>5.63</td>
<td>152.95</td>
<td>Neg</td>
<td>0.9991</td>
<td>0.5958</td>
<td>100-4000</td>
<td>25.8 / 85.9</td>
<td>100.2</td>
<td>5.1</td>
<td>117±6</td>
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<td>Tannic acid</td>
<td>6.46</td>
<td>182.95</td>
<td>Neg</td>
<td>0.9955</td>
<td>0.9075</td>
<td>100-4000</td>
<td>10.2 / 34.2</td>
<td>97.8</td>
<td>5.1</td>
<td></td>
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<td>8</td>
<td>tr-Caffeic acid</td>
<td>7.37</td>
<td>178.95</td>
<td>Neg</td>
<td>0.9942</td>
<td>1.0080</td>
<td>25-1000</td>
<td>4.4 / 14.7</td>
<td>98.6</td>
<td>5.2</td>
<td>32±2</td>
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<td>Vanillin</td>
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<td>151.05</td>
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<td>0.4094</td>
<td>250-1000</td>
<td>10.1 / 33.7</td>
<td>99.2</td>
<td>4.9</td>
<td></td>
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<tr>
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<td>p-Coumaric acid</td>
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<td>162.95</td>
<td>Neg</td>
<td>0.9909</td>
<td>1.1358</td>
<td>100-4000</td>
<td>15.2 / 50.8</td>
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<td>5.1</td>
<td>112±6</td>
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<td>Rosmarinic acid</td>
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<td>358.9</td>
<td>Neg</td>
<td>0.9992</td>
<td>0.5220</td>
<td>250-1000</td>
<td>10.4 / 34.8</td>
<td>101.7</td>
<td>4.9</td>
<td></td>
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<td>12</td>
<td>Rutin</td>
<td>10.18</td>
<td>609.1</td>
<td>Neg</td>
<td>0.9971</td>
<td>0.8146</td>
<td>250-1000</td>
<td>17.0 / 56.6</td>
<td>102.2</td>
<td>5.0</td>
<td>23±1</td>
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<tr>
<td>13</td>
<td>Hesperidin</td>
<td>9.69</td>
<td>611.1 Poz</td>
<td>Neg</td>
<td>0.9973</td>
<td>0.1363</td>
<td>250-1000</td>
<td>21.6 / 71.9</td>
<td>100.2</td>
<td>4.9</td>
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<td>14</td>
<td>Hyperoside</td>
<td>10.43</td>
<td>463.1</td>
<td>Neg</td>
<td>0.9549</td>
<td>0.2135</td>
<td>100-4000</td>
<td>12.4 / 41.4</td>
<td>98.5</td>
<td>4.9</td>
<td>170±8</td>
</tr>
<tr>
<td>15</td>
<td>4-OH Benzoic acid</td>
<td>11.72</td>
<td>136.95</td>
<td>Neg</td>
<td>0.9925</td>
<td>1.4013</td>
<td>25-1000</td>
<td>3.0 / 10.0</td>
<td>106.2</td>
<td>5.2</td>
<td>17±1</td>
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<td>16</td>
<td>Salicylic acid</td>
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<td>136.95</td>
<td>Neg</td>
<td>0.9904</td>
<td>0.6619</td>
<td>25-1000</td>
<td>4.7 / 13.3</td>
<td>106.2</td>
<td>5.0</td>
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<td>17</td>
<td>Myricetin</td>
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<td>317</td>
<td>Neg</td>
<td>0.9991</td>
<td>2.8247</td>
<td>100-4000</td>
<td>9.9 / 32.9</td>
<td>106.0</td>
<td>5.9</td>
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<td>Fisetin</td>
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<td>284.95</td>
<td>Neg</td>
<td>0.9988</td>
<td>2.4262</td>
<td>100-4000</td>
<td>10.7 / 35.6</td>
<td>96.9</td>
<td>5.5</td>
<td></td>
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<td>Coumarin</td>
<td>12.52</td>
<td>146.95</td>
<td>Neg</td>
<td>0.9924</td>
<td>0.4203</td>
<td>100-4000</td>
<td>9.1 / 30.4</td>
<td>104.4</td>
<td>4.9</td>
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<td>20</td>
<td>Quercetin</td>
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<td>300.9</td>
<td>Neg</td>
<td>0.9995</td>
<td>4.3149</td>
<td>25-1000</td>
<td>2.0 / 6.8</td>
<td>98.9</td>
<td>7.1</td>
<td></td>
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<td>21</td>
<td>Naringenin</td>
<td>14.66</td>
<td>270.95</td>
<td>Neg</td>
<td>0.9956</td>
<td>2.0200</td>
<td>25-1000</td>
<td>2.6 / 8.8</td>
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<td>Hesperetin</td>
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<td>0.9961</td>
<td>1.0164</td>
<td>25-1000</td>
<td>3.3 / 11.0</td>
<td>102.4</td>
<td>5.3</td>
<td></td>
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<tr>
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<td>Luteolin</td>
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<td>284.95</td>
<td>Neg</td>
<td>0.9992</td>
<td>3.9487</td>
<td>25-1000</td>
<td>5.8 / 19.4</td>
<td>105.4</td>
<td>6.9</td>
<td></td>
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<td>Neg</td>
<td>0.9917</td>
<td>0.5885</td>
<td>25-1000</td>
<td>2.0 / 6.6</td>
<td>99.1</td>
<td>5.2</td>
<td>49±3</td>
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<td>Apigenin</td>
<td>17.31</td>
<td>268.95</td>
<td>Neg</td>
<td>0.9954</td>
<td>0.6782</td>
<td>25-1000</td>
<td>1.0 / 3.0</td>
<td>98.9</td>
<td>5.3</td>
<td>36±2</td>
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<td>26</td>
<td>Rhamnetin</td>
<td>18.94</td>
<td>314.95</td>
<td>Neg</td>
<td>0.9994</td>
<td>2.5678</td>
<td>25-1000</td>
<td>0.2 / 0.7</td>
<td>100.8</td>
<td>6.1</td>
<td></td>
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<td>27</td>
<td>Chrysan</td>
<td>21.18</td>
<td>253</td>
<td>Neg</td>
<td>0.9965</td>
<td>1.5530</td>
<td>25-1000</td>
<td>0.05 / 0.17</td>
<td>102.2</td>
<td>5.3</td>
<td></td>
</tr>
</tbody>
</table>

*aRT*: Retention time

*Parent ion (m/z)*: Molecular ions of the standard compounds (mass to charge ratio)

*R*: coefficient of determination

*RSD*%: relative standard deviation

*LOD/LOQ* (µg/L): Limit of detection/Limit of quantification

*U* (%): Percent relative uncertainty at 95% confidence level (k=2).

*Amount*: Quantitative phenolic acid composition of *S. lavandulifolia* (ppb; µg analyte/kg extract)
Figure 1. UHPLC-ESI-MS/MS chromatograms of 250 ppb standard mix

Figure 2. UHPLC-ESI-MS/MS chromatograms of S. lavandulifolia.

Antioxidants prevent negative effects of radical species due to their radical scavenging properties. Antioxidant compounds donate hydrogen atoms or transfer single electrons to the radical species to produce nonradical forms [23]. Most of plants have plenty amount of antioxidant potentials and consuming them on diet can prevent some degenerative diseases such as cardiovascular problems, cancer, diabetes, arthritis, and atherosclerosis. Investigation of natural antioxidant sources became very popular research topic in last decades [24].

The ferric thiocyanate method was the one of in vitro antioxidant method that we tried to analyze antioxidant potential of S. lavandulifolia. The method measures the amount of peroxide produced during the initial stages of oxidation which are the primary products of oxidation. The samples (WS and ES) exhibited effective antioxidant activity in the linoleic acid emulsion system. Peroxidation of linoleic acid emulsion without samples or standard compounds was accompanied by a rapid increase in peroxides. Consequently, these results clearly indicate that WS and ES had effective and potent antioxidant activity in the ferric thiocyanate assays. The effect of 20 µg/mL extracts and standards on lipid peroxidation of a linoleic acid emulsion is shown in Fig. 3.
The antioxidant potentials of the samples were determined by reducing power assays. According to the data of FRAP method, samples and standard antioxidants reduce the ferric ions to the ferrous ions. The reducing amounts of samples and standards antioxidants in same concentration (30 µg/mL) ordered as BHA > ascorbic acid > BHT > aqueous extract (WS) > ethanol extract (ES), respectively. Fig. 4 demonstrated lower reducing activity of S. lavandulifolia extracts than standards.

**Figure 4. Ferric reducing antioxidant potential of S. lavandulifolia by FRAP method.**

The cupric reducing potentials of S. lavandulifolia extracts were measured by *in vitro* CUPRAC method. The extracts of S. lavandulifolia (WS, ES) and standard antioxidants showed high reducing antioxidant capacities (Fig. 5). Cupric reducing amounts of samples and standard antioxidants in the same concentration (30 µg/mL) ordered as BHA > BHT > ascorbic acid > ethanol extract (ES) > aqueous extract (WS).
ABTS cation radical scavenging level related with antioxidant capacity. ABTS method based on the reduction of cation radicals by antioxidant substances. According to the data of ABTS radical scavenging, decrease on graph indicated scavenging of cation radicals as shown on Fig. 6. The extracts showed effective ABTS radical scavenging activity like standard antioxidants. Also, the ethanol extract (ES) demonstrated higher cation radicals scavenging activity than aqueous extract (WS).

The DPPH method is a common technique for determination of antioxidant capacity that scavenged free radicals. A noteworthy correlation between concentration and antioxidant potential was detected. *S. lavandulifolia* ethanol extract showed effective DPPH radical scavenging activity close to BHT, a standard antioxidant. Also, the aqueous extract showed low-level free radical scavenging effect (Fig. 7).

**Figure 5.** Cupric reducing antioxidant potential of *S. lavandulifolia* by CUPRAC method.

**Figure 6.** Cation radical scavenging activity of *S. lavandulifolia* by ABTS method.
Conclusions

We investigated in vitro antioxidant activities and some organic compounds of *S. lavandulifolia* in this study. The results showed that the extracts of *S. lavandulifolia* have notable antioxidant potential on radical scavenging and reducing capability methods. Furthermore, quinic acid and chlorogenic acid were detected as the main phenolic compounds by LC-MS/MS. correlated with. The rich phenolic content of *S. lavandulifolia* might contributed its high antioxidant and antiradical potential. This study provide an important step for further studies to perform the features of *S. lavandulifolia* on food industry or pharmacology.

Conflict of Interest

No potential conflict of interest was reported.

References


