Identification of nonvolatile phenolic acids and flavonoids with antioxidant activity in Micromeria barbata extract by RF-HPLC

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Abstract
An effective RF-HPLC method was performed for the determination and purification of nonvolatile mixture of phenolic acid and flavonoids contents in Micromeria barbata ethanolic extract. The HPLC was done on Ascentis RF-Amide 25°C using a mixture of diluted orthophosphoric aqueous acid (0.085%) (A)– acetonitrile (B). A gradient elution was conducted starting at 85% and ending with 15% of A after 60 minutes. Identification of phenolic acids and flavonoids was done by comparing their retention times to those of standard compounds. Aluminum chloride and Folin-Ciocalteau spectrophotometry analysis was used for quantification. It was found that the ethanol extract inhibits the DPPH radical with an IC_{50} (26.74 μg/ml) which was significantly less than that of BHT (91.40 μg/ml).

Keywords: RP-HPLC, retention time, calibration curve, phenolic acids, flavonoids, Micromeria barbata, Antioxidant activity

1. Introduction
The genus Micromeria is part of the Labiatae family that grows in the eastern Mediterranean region including Lebanon. Micromeria have shown great potential in treating human diseases such as headache, wounds, skin infections and common cold. The volatile essential oil of Micromeria was tested earlier by our group and has shown significant antioxidant and antimicrobial activities. In the present study, we are analyzing the nonvolatile components. The study of the nonvolatile phenolic compounds is of great interest because of their variable protective activities such as antimicrobial, antiinflammatory and antioxidative. These phenolic compounds were identified using quantitative HPLC where a set of standards of variable concentrations are run. The plant crude extract peaks in the chromatogram were identified by comparing their retention times with those of the standards and quantified using the calibration curves of the corresponding standards.

2. Methods and materials
2.1 Plant material
Micromeria Barbata was collected from Dennieh, North Lebanon in October 2012 and authenticated by Dr. George Thome, Botanist, (National Council for Scientific Research, Lebanon). The samples were dried at room temperature.

2.2 Solvents and reagents
The solvent used for extraction of Micromeria Barbata was analytical grade ethanol; while those used for the HPLC analysis were HPLC grade acetonitrile and Milli-Q (Millipore Australia Pty. Ltd) distilled water.

Aluminum chloride, potassium acetate, FC reagent, Na_{2}CO_{3}, DPPH and the standards Gallic acid, chlorogenic acid, syringic acid, vanillic acid, caffeic acid, hydroxybenzoic acid, sinapic acid, ferulic acid, p-coumaric acid, cinamnic acid, myrcetin, hesperetin, quercetin, naringenin, chrysin and ellagic acid were all purchased from Sigma Aldrich (Steinheim, Germany).

2.3 Sample preparation
The collected sample of Micromeria barbata was percolated with ethanol at room temperature for two days, filtered, and then solvent dried by vacuum evaporation. 10.5 mg of the extract was dissolved in 1 ml of acetonitrile and directly injected to the HPLC.

2.4 Determination of Total Flavonoids
The quantification of the total phenolic content of the extract was done using the aluminum chloride spectrophotometric method as described by Hossain & Rahman (2011) with slight modifications. 0.5 ml of ethanolic extract (2 mg/ml), 0.1 ml of aluminum chloride (10%), 0.1 ml of potassium acetate (1M) and 4.3 ml of distilled water were mixed together then incubated for 30 minutes. The absorbance was measured at 415 nm and Quercetin was used to make the calibration curve. Results were expressed in mg of quercetin/g dry sample.

2.5 Determination of Total Phenolics
The quantification of the total flavonoid content of the extract was done using the aluminium chloride spectrophotometric method based on the procedure described by Barros et al. (2010) with slight modifications. 1 ml of the ethanolic extract (2 mg/ml) was mixed with 0.5 ml of FC reagent (1N) and diluted by 5ml of distilled water. After 5 minutes, 350 μl of Na_{2}CO_{3} 15% was added. The tubes were allowed to stand for 1.5 h in dark for color development. Absorbance was measured at 725 nm and Gallic acid was used to make the calibration curve. Results were expressed in mg of gallic acid/g dry sample.

2.6 HPLC Analysis
A simple and quick reversed phase method for determination of phenolic acids and flavonoids was developed. Chromatography analysis was performed with the use of liquid chromatographic system, which consisted of Prominence Liquid Chromatographic Shimadzu instrument with UV-Detector-SPD-20 A. The separation was carried out on Ascentis RP-Amide (15 cm x 4.6 mm ID, 5 μm particles) reversed phase column. Column temperature was maintained at 25°C. The mobile phase was a gradient elution of water containing 0.085%
orthophosphoric acid (solvent A) and acetonitrile (solvent B) at a flow rate 1 ml/min. The gradient program of solvent A in B (v/v) was as follows: 0 – 30 min 85 % A; 30 – 35 min 65 % A; 35 – 60 min 15 % A. The injection volume for all samples was 20 µl. For detection, chromatograms were monitored at 280 nm. Identification of phenolic acids and flavonoids was based on retention times in comparison with standards. The quantification was carried out using the external standard method. Stock solution of standard compounds at concentration 1 mg/ml each was prepared in acetonitrile, and several dilutions with acetonitrile were made. The solution of standards at various concentrations (25-125 mg/L) was injected into the HPLC system and the calibration curves were established for each standard compound. The concentration of the compound was calculated from peak area according to calibration curves. The amount of each phenolic acid and flavonoid was expressed as milligram per gram dry sample.

2.7 Antioxidant Activity of the Ethanolic Extract

The radical scavenging activity of the BHT and a prepared solution of the ethanolic extract (S<sub>2</sub>=2mg/ml) were tested using the DPPH radical scavenging activity assay. DPPH radical (1.75 mM) was mixed with a range of (20 µl – 200 µl) of S<sub>2</sub> and the total volume was adjusted to 4 ml by ethanol leading to a range of 0.01 - 0.1 mg/ml solutions of ethanolic extract. The reaction mixture was shaken and then incubated at room temperature in dark for 45 minutes. The DPPH radical inhibition was measured at 517 nm by using a Shimadzu UV spectrophotometer. Using the same conditions, BHT was used as a reference to compare its results to those of the ethanolic extract.

3. Results and Discussion

3.1 Total phenolic and total flavonoid content

The calibration curves of Gallic acid and Quercetin showed linearity of the method over the concentration range analyzed with values of correlation coefficient ‘R’ of 0.9971 and 0.9939 respectively (Figure 1a, Figure 1-b). Using the calibration curves, the total phenolic content of the <i>Micromeria barbata</i> is 60 mg gallic acid/g extract whereas its total flavonoid content is 27.02 mg quercetin/g extract. The results show that the berries contain significant amounts of phenolic compounds where almost half of them are flavonoids.

![Figure 1: Calibration curves of Gallic acid (a) and Quercetin (b)](image)

3.2 HPLC Analysis

Results of HPLC analysis (Figure 2) of the ethanolic extract of Micromeria barbata shows the presence of various constituents as evidenced by the chromatogram obtained at various retention times (1.828, 2.224, 8.044, 9.157, 9.686, 12.339, 14.147, 16.778, 17.521, 30.516, 33.828, 36.254, 36.775, 37.147, 40.142). The chemical composition and the retention time of the ethanol extract are summarized in Table 1. The main phenolic acids identified were chlorogenic (7.409 mg/g of extract) and the main flavonoids were myricetin (4.095 mg/g of extract), hesperitin (7.52 mg/g of extract) and quercetin (4.148 mg/g of extract). These results summarized in Table 1 are in accordance with the total phenolic and flavonoidal content of the extract where the flavonoids represent approximately half of the total phenolic content. The significant amounts of chlorogenic acid and hesperitin that were detected in the extract can be attributed to possible usage of this herb in the treatment of certain diseases. Chlorogenic acid plays a great role in preventing various diseases associated with oxidative stress such as cancer, cardiovascular, aging and neurodegenerative disease<sup>12,13</sup> and hesperitin, myricetin and quercetin possess antimutagen and anticarcinogen activities.<sup>13</sup>

<table>
<thead>
<tr>
<th>Compound</th>
<th>mg/g of extract</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Gallic acid</td>
<td>0.332</td>
<td>1.828</td>
</tr>
<tr>
<td>2-Chlorogenic acid</td>
<td>7.409</td>
<td>2.224</td>
</tr>
<tr>
<td>3-Syringic acid</td>
<td>0.246</td>
<td>8.044</td>
</tr>
<tr>
<td>4-Vanilic acid</td>
<td>1.01</td>
<td>9.157</td>
</tr>
<tr>
<td>5-Caffeic acid</td>
<td>0.149</td>
<td>9.686</td>
</tr>
<tr>
<td>6-Hydroxybenzoic acid</td>
<td>1.299</td>
<td>12.339</td>
</tr>
<tr>
<td>7-Sinapic acid</td>
<td>1.070</td>
<td>14.147</td>
</tr>
<tr>
<td>8-Ferric acid</td>
<td>0.597</td>
<td>16.778</td>
</tr>
<tr>
<td>9-p-Coumaric acid</td>
<td>0.092</td>
<td>17.521</td>
</tr>
<tr>
<td>10-Cinnamic acid</td>
<td>0.066</td>
<td>30.516</td>
</tr>
<tr>
<td>11-Myricetin</td>
<td>4.095</td>
<td>33.828</td>
</tr>
<tr>
<td>12-Hesperetin</td>
<td>17.53</td>
<td>36.754</td>
</tr>
<tr>
<td>13-Narigenin</td>
<td>1.223</td>
<td>36.775</td>
</tr>
<tr>
<td>14-Quercetin</td>
<td>4.148</td>
<td>37.147</td>
</tr>
<tr>
<td>15-Ellagic acid</td>
<td>0.549</td>
<td>40.142</td>
</tr>
</tbody>
</table>
Figure 2: HPLC chromatogram of the ethanolic extract of *Micromeria Barbata*


3.3 Antioxidant capacity

The degree of inhibition was calculated using the following equation:

\[
\text{% DPPH Inhibition} = \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \times 100
\]

The ethanolic extract of the *Micromeria barbata* inhibited the activity of DPPH radical in a dose response relationship (Figure 3). In this case, the IC\(_{50}\) of the extract was 26.74μg/ml which was significantly less than that of BHT (91.40 μg/ml). So the ethanolic extract is a better antioxidant than the commonly used synthetic BHT. This can be explained by the elevated levels of chlorogenic acid which were detected in the extract along with other phenolic compounds (Table 1). Chlorogenic acid has strong antioxidant properties which are suggested to play an important role in protecting food, cells and any organ from oxidative degenerative.\(^{14,15}\)

Figure 3: % of DPPH Inhibition of the Ethanolic Extract

4. Conclusion

The ethanolic extract of *Micromeria barbata* is rich in phenolic acids like chlorogenic acid and flavonoids like hesperitin myrcetin and quercetin. These phenolic compounds were shown to be effective against a wide range of diseases as they possess antimutagenic, anti-cancer and antioxidative activities. The results of the DPPH radical scavenging activity of the extract revealed its prevalence over the commonly used synthetic antioxidant. This shows that the herb can be used as an effective natural food additive in order to preserve food quality and decrease the oxidative stress.

References