Determination of Total Phenolic and Flavonoid Contents of
Leonurus cardiaca L. in Compare with Antioxidant Activity

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Abstract: The crude extract and 4 fractions of aerial parts of cultivated motherwort (Leonurus cardiaca L.) were investigated for their total phenolic and flavonoid contents and antioxidant activities. The phenolic and flavonoid contents were determined using Folin-Ciocalteu assay and proposed assay of pharmacopeia where as antioxidant activity of samples were measured by 2 tests: DPPH and FRAP assays. Butanolic fraction showed the highest antioxidant activity with the FRAP value of 1.735±0.07 μmol g⁻¹ and DPPH inhibitory percentage of 57.76% μg mL⁻¹ as well as the greatest phenolics content (48.37±3.76 mg of gallic acid equivalents g⁻¹). The highest total flavonoid content was revealed in methanolic-aqueous fraction (50.21± 0.65 mg of hyperoside equivalents g⁻¹). There was a direct correlation between total phenol and antioxidant activities which could introduce phenols as the main antioxidants of L. cardiaca L. extracts.

Key words: Leonurus cardiaca L., antioxidant, phenolic content, flavonoid, methanolic-aqueous, fraction, hyperoside equivalents

INTRODUCTION

In recent years, the possible role of nutrition in prevention of human ailments has been shown to be important. In this context, antioxidants, especially those derived from natural sources, require special attention. These beneficial compounds neutralize the toxic and volatile free radicals that are defined as atoms or groups of atoms having an unpaired electron (Hiramatsu et al., 2006). Most antioxidants in plants are phenols which act as chain-breaking antioxidants. Phenols sometimes have additional mechanisms of antioxidant action, e.g., by chelating transition metal ions (Packer and Cadenas, 2002). Flavonoids as the largest group of phenols consist of an aromatic ring, which is condensed to a heterocyclic ring and attached to a second aromatic ring. The abundant phenolic hydroxyl groups on the aromatic ring confer the antioxidant capacity and the 3-OH is essential for the iron-chelating activity of these compounds (Hiramatsu et al., 2006). Cellular damage induced by oxidative stress, a result of imbalance between the antioxidant defense system and the formation of reactive oxygen species has been implicated in the etiology of a large number of human diseases as well as in the process of aging. The degenerative diseases in which free radicals have been implicated include cardiovascular ailments, neurodegenerative diseases such as Parkinson’s disease, Alzheimer’s disease and multistage process of carcinogenesis (Hiramatsu et al., 2006; Delghan et al., 2007). Recently, there have been great efforts in finding safe and potent natural antioxidants from various plant sources to replace synthetic ones.

Motherwort (Leonurus cardiaca L.) which originally came from central Europe, has spread to all temperate areas of the world. It has been listed in many pharmacopeias including the current European Pharmacopoeia. European Motherwort is stated to possess efficient antioxidant properties (Matkowski and Piotrowska, 2006; Matkowski et al., 2008) and known to have significant amounts of phenolics and flavonoids (Barnes et al., 2007). Traditionally, it has been used for cardiac debility, simple tachycardia, effort syndrome, amenorrhoea and especially for cardiac symptoms associated with neurosis (Gruenwald et al., 2000).

In Iran, L. cardiaca is cultivated for the first time in order to produce medicinal formulations whereas it has been approved that changing in growth condition can make a variation in the plant main components and consequently its medicinal effects (Urban et al., 2009). The present study leads to evaluate, different extracts of cultivated motherwort for their antioxidant activities using two methods: inhibition of DPPH radical and FRAP. Also, the total phenolic and flavonoid contents of the samples were estimated using the Folin-Ciocalteu assay and proposed method of European pharmacopeia, respectively.

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MATERIALS AND METHODS

Plant material: The aerial parts of Leonurus cardiaca L. were obtained from the experimental plot of the Zardband Pharmaceuticals, Tehran, Iran in July 2009 during the flowering stage. The voucher specimen was deposited in their Herbarium (No. 16 ZA 0526-07).

Chemicals: All reagents and solvents were of analytical grade or of pure quality which were purchased from Merck, Sigma, Aldrich and Fluka.

Extraction: The extract was obtained by percolation using methanol/H2O (80:20, v/v) at room temperature. The procedure involved 3 consecutive extractions of 48 h using new solvent each time. The solvent was evaporated under vacuum in a rotary evaporator until dryness. The dried extract fractionated using chloroform, ethylacetate, n-butanol and methanol/H2O (50:50, v/v) successively. Then dried total extract and fractions were stored at 20°C until use.

Measurement of total phenolics: Total phenolic content was determined spectrophotometrically using Folin-Ciocalteau reagent (Dehghan et al., 2007). Folin-Ciocalteau reagent (0.75 mL), previously diluted 10-fold with distilled water were mixed thoroughly with 200 μL of appropriate dilutions of the crude extract or fraction solutions and allowed to stand at room temperature for 5 min. Then 0.75 mL of sodium bicarbonate solution (60 g L−1) was added to the mixture followed by storing at room temperature for 90 min. Subsequently, absorbance was measured at 725 nm using a UV-visible spectrophotometer. Total phenolics were quantified by calibration standard curve obtained using various known concentrations of gallic acid (50-200 μg mL−1 in methanol). The concentrations are expressed as mg of Gallic Acid Equivalents (GAE) g−1 of dry extract or fraction. The total phenolics content of samples was measured 5 times for each species.

Measurement of total flavonoids: The total flavonoid content of the extract and fractions were determined using proposed method of Council of Europe (2005). Total flavonoid contents were expressed as mg of Hyperoside Equivalents (HE) per g of sample.

DPPH radical scavenging activity: The DPPH radical scavenging activity was measured using the method described by Sanchez-Moreno et al. (1999) with some modification. Total extract and its fractions were dissolved in methanol at different concentrations (50, 100, 200 and 500 mg = mL). About 1 mL of these solutions was added to 2 mL of DPPH solution (4·10−3 g mL−1 in methanol). Absorbance was measured immediately after mixing the solutions at 517 nm using methanol as blank and the absorbance decrease was evaluated every 5 min up to 30 min. About 2 mL of DPPH diluted in 1 mL of methanol was used as control.

The scavenging activity was calculated using the following equation: Inhibition (%) = 100 - [(A0 - As)/A0] × 100 where A0 is the absorbance of the control (The DPPH solution without sample solution) and as is the absorbance in the presence of sample. IC50 value was determined from the plotted graph of scavenging activity against the concentrations of the samples which represented the total antioxidant necessary to decrease the initial DPPH radical by 50%. α-tocopherol and BHA were used as positive control and all experiments were carried out at least 3 times.

Frap assay: The ferric-reducing ability of extract and fractions were determined at low pH by Frap assay (Benzie and Strain, 1996; Astaneh et al., 2005). Briefly, the FRAP reagent contained 5 mL of a (10 mmol L−1) TPTZ (2, 4, 6-tripyridyl-s-trazine) solution in 40 mmol L−1 HCl plus 5 mL of (20 mmol L−1) FeCl3 and 50 mL of (0.3 mol L−1) acetate buffer, pH 3.6 and was prepared freshly and warmed at 37°C. Aliquots of 100 μL sample were mixed with 3 mL FRAP reagent and the mixtures were incubated at 37°C for 10 min. An intense blue color complex was formed when Fe³⁺ TPTZ complex was reduced to the Fe²⁺ form and the absorption at 593 nm was measured. The calibration curve was plotted with absorbance at 593 nm vs. About 5 different concentrations of FeSO4·7H2O (125, 250, 500, 750, 1000 μmol L−1). All solutions were used on the day of preparation and the results were expressed in mmol Fe²⁺ g−1 of extract or fraction.

Statistical analysis: Values were reported as mean±SD of three parallel measurements. The obtained results were statistically analyzed with one way ANOVA and Tukey post hoc multicomparison tests using a significance level of p<0.05. Correlation between phenolic and flavonoid contents and antioxidant activities was established by regression analysis.

RESULTS

Fractionation: Total amounts of crude extract and fractions are shown in Table 1. The amount of Methanolic-aqueous was considerably noticeable among other fractions.
Table 1: Total amounts of the motherwort crude extract and fractions

<table>
<thead>
<tr>
<th>Yield</th>
<th>Total extract</th>
<th>Chlороform</th>
<th>Ethyl acetate</th>
<th>n-Butanol</th>
<th>Methanolic-aqueous (50:50, v/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total amount (yielded by 100 g of dry plant)</td>
<td>31.78</td>
<td>1.82</td>
<td>1.13</td>
<td>5.55</td>
<td>22.30</td>
</tr>
<tr>
<td>Percentage (relative to crude extract)</td>
<td>100.00</td>
<td>5.73</td>
<td>3.55</td>
<td>17.42</td>
<td>66.83</td>
</tr>
</tbody>
</table>

Table 2: Total phenolic and flavonoid contents and antioxidant activity of motherwort crude extract and fractions

<table>
<thead>
<tr>
<th>Samples</th>
<th>Phenolics content (mg GAE g⁻¹±SD)</th>
<th>Flavonoids content (mg HE g⁻¹±SD)</th>
<th>DPPH (% inhibition±SD)</th>
<th>IC₅₀ (µg mL⁻¹)</th>
<th>FRAP value (mmol Fe²⁺ g⁻¹±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroform</td>
<td>4.90±0.98</td>
<td>27.25±0.670</td>
<td>Trace</td>
<td>1814±35</td>
<td>1.23±0.05</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>20.45±2.54</td>
<td>0.42±16.27</td>
<td>47.20±3.87</td>
<td>107.16</td>
<td>0.42±0.03</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>48.37±3.76</td>
<td>0.20±8.750</td>
<td>86.18±5.76</td>
<td>57.76</td>
<td>1.73±0.07</td>
</tr>
<tr>
<td>Methanolic-aqueous</td>
<td>42.95±3.55</td>
<td>0.65±50.21</td>
<td>85.84±6.76</td>
<td>53.79</td>
<td>1.52±0.05</td>
</tr>
<tr>
<td>Total extract</td>
<td>62.85±3.70</td>
<td>0.05±14.35</td>
<td>80.59±6.98</td>
<td>77.65</td>
<td>1.10±0.06</td>
</tr>
</tbody>
</table>

GAE = Gallic Acid Equivalent; SD = Standard Deviation; HE = Hydroxy Equivalent; IC₅₀ = 50% Inhibitory Concentration; The reported percentages for % inhibition were related to the concentration of 100 µg extract or fraction mL⁻¹

Total phenol estimation: The total phenolic contents of the samples were calculated with a linear equation based on a standard curve using gallic acid \(y = 0.005x + 0.0468, R^2 = 0.9987\) and shown in Table 2. The buthanolic fraction had the highest phenolic content (48.37±3.76 mg GAE g⁻¹).

Flavonoid content: According to the quantitative results shown in Table 2, there are marked differences between fractions in total flavonoid contents. The highest amount of flavonoid was found in methanalic-aqueous fraction (50.2±0.65 mg HE g⁻¹) while buthanolic fraction was determined as the lowest (8.75±0.20 mg HE g⁻¹).

DPPH radical scavenging activity: The results of the DPPH assay are shown in Table 2. The higher polar fractions, methanalic-aqueous and buthanolic ones showed high antioxidant activity. Radical scavenger activity of these two fractions at 75 µg mL⁻¹ were comparable with α-tocopherol (40 µg mL⁻¹) and BHA (100 µg mL⁻¹), p<0.05.

FRAP assay: Antioxidant activity of all samples were calculated with a linear equation based on a standard curve using FeSO₄ \(y = 0.0007x + 0.0589, R^2 = 0.9512\) and shown in Table 2. The buthanolic fraction showed the highest antioxidant activity with the FRAP value of 1.735±0.07 mmol g⁻¹.

DISCUSSION

The amounts of dry extract and fractions obtained from aerial part of *L. cardiaca* L. are shown in Table 1. These results showed that polar solvents could produce massive fractions with most of the constituents. Also, study of estimated total phenolic contents (Table 2) reveals that phenolic components in non polar solvents (chloroform and ethyl acetate) are noticeably less than crude extract and polar fractions (buthanolic and methanolicaqueous).

So, non polar solvent are not efficient enough for extraction of Motherwort. In previous investigations, the presence of various polyphenol compounds with antioxidant activity were reported in Motherwort (Ali et al., 2007; Bernatoniene et al., 2009).

High phenolic contents were previously reported in buthanolic (256.8±7.8 GAE mg g⁻¹) and methanalic (150.1±8.9 GAE mg g⁻¹) extracts (Matkowski et al., 2008). In another study, IC₅₀ of methanalic extract of *L. cardiaca* was reported about 70-80% (Matkowski and Pietrowska, 2006) which is approximately comparable with the results in crude extract whereas buthanolic and methanalicaqueous fractions of the research were more potent in IC₅₀ assay (53.79% and 57.76%, respectively). Total flavonoid content of crude extract was estimated about 0.45% of flavonoids expressed as hydroxy in dried plant (equal to 14.35 mg HE g⁻¹ of total extract) which is remarkably higher than minimum approved flavonoid content of Motherwort reported in pharmacopeias (0.2% of dried plant).

The results showed that omission of chloroform fraction reports could lead to a significant correlations between antioxidant activities, phenolic and flavonoid contents in polar and semi-polar fractions of motherwort (Table 3) and phenolic compounds of this plant are probable responsible compounds for antioxidant power. It seems higher antioxidant activity and total phenolic content were exhibited in methanalic-aqueous and buthanolic fractions whereas massive methanalicaqueous fraction is considerable for further studies.
Table 3: Correlations between antioxidant activities and Phenolic and flavonoid contents of fractions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phenolic content (mg GAE g⁻¹) and FRAP value (mmol Fe⁺ g⁻¹)</th>
<th>DPPH content (%) inhibitory and DPPH value (mmol Fe⁺ g⁻¹)</th>
<th>Phenolic content (mg GAE g⁻¹) and flavonoid content (mg RE g⁻¹) and FRAP value (mmol Fe⁺ g⁻¹)</th>
<th>Flavonoid content (mg HE g⁻¹) and DPPH content (%) inhibitory and DPPH value (mmol Fe⁺ g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equation</td>
<td>$y = 0.6476x - 0.3473$</td>
<td>$y = 1.4899x + 17.564$</td>
<td>$y = 0.0311x - 1.0483$</td>
<td>$y = 0.0245x + 1.5877$</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.9991</td>
<td>0.9692</td>
<td>0.9788</td>
<td>0.9269</td>
</tr>
</tbody>
</table>

CONCLUSION

Motherwort used for a variety of human diseases, especially cardiac disorders. This plant is cultivated in Iran for the first time in order to use in medicinal formulations.

In summary, the current study verifies high amount of flavonoid in total extract and fractions of cultivated *L. cardiaca* which can offer the plant for industrial uses.

ACKNOWLEDGEMENTS

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REFERENCES


