Alfa-Glucosidase inhibitory and antioxidant activity of hexane extract of flowers, leave and stems of *Haplophyllum acutifolium* DC. and *Ferula haussknechtii* Wolff ex Rech.

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**ABSTRACT**

**Background:** α-Glucosidase inhibition can significantly prevent glucose uptake after meal, and help controlling adverse effects in diabetics. So, determination of α-Glucosidase inhibitory effect and antioxidant activity of the *Haplophyllum acutifolium* and *Ferula haussknechtii* aerial organs was the aim of this study.

**Methods:** Inhibitory effect of hexane extracts from different organs was investigated spectrophotometricaly in several concentrations at 405 nm wavelength using a microplate reader. Antioxidant activity of hexane extracts from various organs was also measured using DPPH scavenging and iron reduction tests.

**Results:** The highest inhibitory activity of *F. haussknechtii* was observed at the 0.1 g/ml concentration of flower extract (100% inhibition and IC\textsubscript{50} = 0.1 μg/ml) and the most inhibitory activity of *H.acutifolium*, was related to the 1 g/ml concentration of flower extract (100% inhibition and IC\textsubscript{50} = 10 μg/ml) and leaf extract (100% inhibition and IC\textsubscript{50} = 60 μg/ml). Extract of *H. acutifolium* flower and leaves showed Non-competitive inhibition pattern and *F. haussknechtii* flower showed mixed (Competitive-Non-Competitive) inhibitory pattern at 0.001 g/ml and exhibit uncompetitive inhibitory pattern at the 0.1 g/ml. The results of antioxidant potential showed EC\textsubscript{50} for *F. haussknechtii* flower and *H. acutifolium* leaves equalled 2.37 and 0.96 mg/ml, respectively.

**Conclusions:** The hexane extract of the *F. haussknechtii* flower, and *H. acutifolium* flower and leaf organ have a significant inhibitory effect on the activity of α-Glucosidase, DPPH free radical scavenging activities and reducing power. So, they are good resources for extraction of medicinal compounds to control blood level of glucose after meal, in diabetic patients.

**Key words:** Alpha-glucosidase, *Ferula haussknechtii*, *Haplophyllum acutifolium*, Diabetes, Enzyme inhibition
Introduction

Diabetes mellitus (DM) is a metabolic disturbance (Bachhawat et al., 2011) that is distinguished by high blood glucose levels and lack or insufficient insulin secretion (Becerra-Jiménez and Andrade-Cetto, 2012). The hyperglycemia is related to atherosclerotic disease (Giacco and Brownlee, 2010) and enhanced occurrence of microvascular complication in diabetic people (Shim et al., 2003). The chronic hyperglycemia is able to stimulate the non-enzymatic glycosylation of several proteins (Lebovitz, 2001) which results in their dysfunction by disordered molecular development and modify enzymatic function (Singh et al., 2014). Several pharmacological methods have been reported for treatment of diabetes, such as α-glucosidase inhibition (Misbah et al., 2013). α-glucosidase inhibitors (AGIs) postpone polysaccharides digestion and delay glucose uptake, thus reducing blood glucose levels (Lawag et al., 2012). Reactive oxygen species (ROS) are the results of common metabolism and have useful or damaging effects (Fatehi-Hassanabad et al., 2010). Several pieces of evidence suggest that long time exposure to chronic glucose level stimulates the generation of free radicals, especially ROS (Bonnefont-Rousselot et al., 2000). It also indicated that oxidative damage via ROS induces some complications in diabetes (Rains and Jain, 2011). Diabetes also changes the activity of antioxidant enzymes in some diabetic tissues, such as the liver, aorta, and kidneys (Hünkar et al., 2002). Any disturbances in the balance among ROS and antioxidant agents may result in a destructive process and damage to cell components (Calabrese et al., 2007). Numerous pharmaceutical herbs have been consumed for many years to control diabetes in different countries. Iran is a country with a great diversity in plant species, and these varieties of plants are scattered across different regions (Sadat-Hosseini et al., 2017). So, it is a good resource for plants with pharmaceutical effects. The α-glucosidase inhibitory of Ferula haussknechtii, and Haplophyllum acutifolium, have been reported earlier (Zarei and Poursharifi, 2015). The aim of this research was to determine the potential α-glucosidase inhibitory and antioxidant activity of hexane extracted from separated different aerial parts of these plants.

Experimental

Plant materials

Plants aerial parts were collected from the central areas of Kurdistan Province, Iran, from June to July 2017. Voucher specimens have been deposited in the Herbarium of the Institute of Agricultural Research. Their flowers, leaves and stems were separated from each other as soon as possible. Separated organs were dried out under shadow and ground by electric grinder to a fine powder.

Preparation of crude extract

About 40 g of each ground powder was soaked in 200 ml hexane for 72 h with sporadic shaking. The soaked substance was filtered throughout Whatman grade No. 42, filter paper. The solvent was evaporated with rotary evaporator. The residues were air dried under chemical hood and finally collected in small microfuge tubes and stored at -20 °C. For better solubility in water-based reaction media, aerial parts 10 mg of hexane
extracts were weighed and dissolved in 10 ml DMSO. This stock solution was diluted to different concentrations and used for further steps.

**Inhibition assay for yeast α-glucosidase activity**

In this study, to evaluate the inhibitory effect of plant extracts on the alpha-glucosidase enzyme, Pistia-Brueggeman method was used with a few changes (Pistia-Brueggeman and Hollingsworth, 2003). 96-well microplate with 150 μl final reaction mixture volume for each well, containing 50 μl of phosphate buffer (50 mM; pH 6.8), 10 μl of alpha-glucosidase (1 U/ml) and 20 μl of plant extract with various concentrations was incubated for 5 min at 37 °C, then 20 μl of 1 mM p-Nitrophenyl α-D-Glucopyranoside (pNPG) substrate was added to the mixture. After additional incubation at 37 °C for 30 min, the reaction was stopped by addition of 50 μl of 0.1M Na₂CO₃. Enzyme, inhibitors and substrates were dissolved in the same buffer (50 mM; pH 6.8). Acarbose was used as the positive control. Each experiment was performed in three replications with appropriate blanks. The percentage of inhibition of alpha-glucosidase enzyme by extract was obtained, using the following formula.

\[
\% \text{ Inhibition} = \frac{\text{Absorbance (Control)} - \text{Absorbance (Extract)}}{\text{Absorbance (Control)}} \times 100
\]

The IC₅₀ value, defined as the concentration of the sample to inhibit 50% of α-glucosidase activity, was estimated using nonlinear regression analysis using Sigma Plot software.

**Kinetic analysis of α-glucosidase inhibition**

Using a range of final assay plant extract concentrations (200, 100, 50, 10, 1, 0.1, 0.01, 0.001, 0.0001 mg/ml) and six pNPG substrate concentrations (0.311, 0.622, 1.24, 1.87, 2.48 and 3.11 mM), Lineweaver-Burk plots were drawn. Using those plots, Vₘₐₓ and Kₘ values were calculated for each inhibitor concentration. Secondary plots, 1/slope of Lineweaver-Burk trend line versus inhibitor concentration, and 1/Vₘₐₓ versus inhibitor concentration, permitted Kᵢ and Kᵢᵠ calculation, respectively.

**Antioxidant activity**

To evaluate the antioxidant activity of plant extracts, two common methods including free radical DPPH scavenging potential and iron reducing power were used. The first approach is to trap the DPPH radicals by the ability to hydrogen donation by the extract (Chung *et al.*, 2006). 1,1-diphenyl-2-picrylhydrazyl (DPPH) is a violet stable radical which by reducing by an electron-donor agent (antioxidant compounds such as ascorbic acid), turns into yellow non-radical diphenylpicrylhydrazine (Molyneux, 2004). In the second approach, the reducing power of the extracts was evaluated by its potency to reduce Ferric iron to its ferrous state. Iron reducing is often used as an electron donating potential measurement, which is a suitable method for evaluating the antioxidant activity of phenolic compound (Hinneburg *et al.*, 2006).
DPPH free radical-scavenging capacity

Free radical scavenging activity was measured according to the method of Rao-Fu et al., with a few modifications (Fu et al., 2014). Briefly, a methanolic solution (0.1 ml) of the sample at different concentrations were added to 0.1 ml of DPPH (0.1 mM) solution. The reaction mixture was shaken and after 30 minutes incubation at room temperature, the absorbance was measured at 517 nm. The percentage (%) of radical scavenging activity (RSA) was determined using the following formula:

\[
\% \text{ RSA} = \left( \frac{\text{Absorbance (Control)} - \text{Absorbance (Extract)}}{\text{Absorbance (Control)}} \right) \times 100
\]

The methanolic DPPH solution was also used as a negative control. Ascorbic acid was used as a positive control with 0.031, 0.25, 0.5, 1, 2, 5, 8 and 12 μg/ml concentrations. EC_{50} values indicate the concentration of the sample needed to scavenge 50% of the DPPH free radicals.

Determination of reducing power

The reduction power of extracts was measured according to the method of Yen and Chen (Yen and Chen, 1995). The extract was prepared with different concentrations of 50, 100, 200, 400, 600 and 800 μg/ml. Then phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%) were added. The mixture was incubated at 45 °C for 30 minutes. At the end of incubation time, 2.5 ml of 10% trichloroacetic acid (TCA) was added to the mixture, and it was centrifuged for 10 minutes at 2383 g. After the end of the centrifugation, 2.5 ml of top solution was mixed with 2.5 ml distilled water and FeCl_3 (0.5 ml, 0.1%) and absorbance was measured at 700 nm.

Statistical analysis

The data and results of the experiments and diagrams in this research were analysed using Sigma Plot software, version 12. One-way analysis of variance (ANOVA) was used to examine and compare the mean of hexane extracts; p<0.05 was considered statistically significant.

Results

Inhibition of α-glucosidase activity

The hexane extracts of *Ferula haussknechtii* flower and *Haplophyllum acutifolium* flower and leaf, showed 100% alpha-glucosidase inhibitory activity at 1000 mg/ml concentration (Table 1). The hexane extract of stem organ from both plants did not show considerable inhibitory effect. Acarbose, an available agent for alpha-glucosidase inhibition showed IC_{50} value of 0.35±0.03 μg/ml. In all of the enzyme activity measurements, acarbose was used as a positive control.
Table 1. The inhibitory effect of aerial parts of plant extracts on α-glucosidase, positive control was Acarbose

<table>
<thead>
<tr>
<th>Botanical name</th>
<th>Family</th>
<th>Organ</th>
<th>Percent inhibition at 10 mg/ml</th>
<th>IC₅₀ (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferula haussknechtii</td>
<td>Apiaceae</td>
<td>Flower</td>
<td>100 ± 0.17</td>
<td>0.001 ± 0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leaf</td>
<td>95 ± 0.12</td>
<td>0.17 ± 0.018</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stem</td>
<td>53.0 ± 0.09</td>
<td>-</td>
</tr>
<tr>
<td>Haplophyllum acutifolium</td>
<td>Rutaceae</td>
<td>Leaf</td>
<td>100 ± 0.13</td>
<td>0.01 ± 0.002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Stem</td>
<td>56.0 ± 0.11</td>
<td>-</td>
</tr>
</tbody>
</table>

Since IC₅₀ for hexane extracts from Ferula haussknechtii flower and Haplophyllum acutifolium flower and leaf, were relatively lower than all other aerial part extracts, so their Lineweaver-Burk (LB) plot were drawn to determine the type of inhibition. The inhibition type on alpha-glucosidase was non-competitive type for Haplophyllum acutifolium flower (Figure 1) and leaf (Figure 2) extracts, and for Ferula haussknechtii was uncompetitive type inhibition for flower (Figure 3) extract, and mixed (Competitive-Non-Competitive) type inhibition for leaf extract (Figure 4). Then Ki (Inhibitor constant) value was determined by plotting slops of primary plots against the inhibitor concentrations. So, Ki for the Ferula haussknechtii flower extract was 2.12 mg/ml (Figure 5) and for Haplophyllum acutifolium leaf extract was 4.76 mg/ml (Figure 6).

Figure 1. Lineweaver-Burk plot for kinetic analysis of α-glucosidase inhibition by Haplophyllum acutifolium flower (10 mg/ml) extracts with pNPG as substrate
Figure 2. Lineweaver-Burk plot for kinetic analysis of α-glucosidase inhibition by *Haplophyllum acutifolium* leaf (10 mg/ml) extracts with pNPG as substrate.

Figure 3. Lineweaver-Burk plot for kinetic analysis of α-glucosidase inhibition by *Ferula haussknechtii* Leaf (10 mg/ml) extracts with pNPG as substrate.

Figure 4. Lineweaver-Burk plot for kinetic analysis of α-glucosidase inhibition by *Ferula haussknechtii* flower (10 mg/ml) extracts with pNPG as substrate.
Figure 5. Secondary plots for the hexane extract of *Ferula haussknechtii* flower to determine the inhibitor constant ($K_i=2.12$ mg/ml). The (-$K_i$) location is shown with an arrow.

Figure 6. Secondary plots for the hexane extract of *Haplophyllum acutifolium* flower to determine the inhibitor constant ($K_i=4.76$ mg/ml). The (-$K_i$) location is shown with an arrow.

**Antioxidant activity**

DPPH radical scavenging activity and $EC_{50}$ values was measured for aerial part *Ferula haussknechtii* and *Haplophyllum acutifolium* (Table 2). The results of reducing power tests showed that these extracts had the ability as electron donors and with increased reducing power, absorption also increases. The reducing power reflects the antioxidant electron donating power. In this method, the reducing power of the hexane extract of aerial organs is evaluated based on the reduction of Fe III to Fe II, and the extract solution of any organ which has high reducing power, showed higher absorbance. Percentages of iron reduction ability for hexane extract of both *Ferula haussknechtii* and *Haplophyllum acutifolium* are shown in Figures 7 and 8.
Table 2. DPPH radical scavenging activity and EC<sub>50</sub> values was measured for aerial part Ferula haussknechtii and Haplophyllum acutifolium

<table>
<thead>
<tr>
<th>Systematic Name</th>
<th>Organ</th>
<th>Reduction%</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferula haussknechtii Wolff ex Rech</td>
<td>Flower</td>
<td>100 ± 0.05</td>
<td>2.37 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>94 ± 0.38</td>
<td>6.31 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>66.66 ± 0.46</td>
<td>28.18 ± 1.4</td>
</tr>
<tr>
<td>Haplophyllum acutifolium DC</td>
<td>Flower</td>
<td>100 ± 0.04</td>
<td>0.96 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>100 ± 0.04</td>
<td>22.39 ± 1.31</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>80.3 ± 0.1</td>
<td>22.39 ± 1.31</td>
</tr>
</tbody>
</table>

Discussion

This study aimed to find natural and safe sources for the isolation and extraction of compounds with alpha-glucosidase inhibitory properties. Detailed studies on two plants, namely Ferula haussknechtii and Haplophyllum acutifolium, were performed. 

Figure 7. Iron reduction potential for hexane extract of Ferula haussknechtii at concentrations of 50-800 mg/ml (ascorbic acid as standard)

Figure 8. Iron reduction potential for hexane extract of Haplophyllum acutifolium at concentrations of 50-800 mg/ml (ascorbic acid as standard)
(Rutaceae) is a genus of perennial small flowering plants containing about 50 species, extending in the eastern Mediterranean from Europe and Africa to the eastern parts of Siberia. There are about 30 species of this species in Iran, of which 14 are native. *Haplophilum* is known as a rich source of quinoline-containing alkaloids. Different extracts of *Haplophilum* are used in traditional medicine as analgesic, antispasmodic, diuretic and sedative as well as topical agents against skin disease. Quinolinated alkaloids isolated from *Haplophilum* and other members of the Rutaceae family have estrogenic, antifungal, antialgae, antiviral, antibacterial and antiparasitic activity (Staerk et al., 2009).

The genus *Ferula* belongs to the Apiaceae family, known as Persian coma, consists of 170 to 187 species (Kurzyna-Młynik et al., 2008). From Central Asia to the west, it spreads across the Mediterranean to North Africa. It is considered to be the largest member of the Apiaceae family in Asia and the third genus of that family in the world (Pimenov and Leonov 2005).

According to the results of this study, the highest inhibitory activity of *Ferula* was related to the of 0.8 g/ml concentration of flower extract (100% inhibition and IC₅₀ equal to 0.7 μg/ml), and the highest inhibitory activity of *Haplophilum* was related to 1 g/ml concentration of flower organ (100% inhibition and IC₅₀ equal to 10 μg/ml) and to 1 g/ml concentration of leaf organ (100% inhibition and IC₅₀ equal to 60 μg/ml). Similar results were gained for rhizome *Picrohiza kurroa* and root of *Rubia cordifolia* (Bachhawat et al., 2011), and for roots of *Cudrania tricuspidata* (Son and Lee 2013).

According to the results of kinetic study of enzyme inhibition using Line weaver-Burk plot, flower and leaf extract of *Haplophyllum acutifolium* showed Non-competitive inhibition, and Leaf extract of *F. vaillantii* showed mixed (competitive-uncompetitive) pattern of inhibition for α-glucosidase.

In uncompetitive inhibition, the inhibitor of the extract binds to the enzyme-substrate complex. Previous substrate binding to the enzyme is probably required to form an inhibitor-binding site on the enzyme. Due to the presence of shared binding sites or spatial interference of these sites in competitive inhibition, there was a competition between the inhibitor and the substrate for binding to the enzyme and only one of them could bind to the enzyme. In the non-competitive inhibition it can be inferred that the inhibitor in the extract binds to both of the enzyme and to the enzyme-substrate complex.

The results of DPPH free radical scavenging experiments by *Ferula* and *Haplophyllum* compared to positive control (ascorbic acid) showed that the anti-radical property of aerial organs was concentration dependent. As the concentrations of each organ extract increased, more free radical scavenging activity was exhibited. The *Ferula* leaf extract scavenging activity at (0.5 g/ml) and its flower extract at (0.5 g/ml) was comparable to ascorbic acid. The results of this study indicated that there is a synergy between the ability of enzyme inhibition and the antioxidant ability of the extracts, as reported before by other researchers (Basak and Candan 2013).

Recent studies have shown that flavonoids are effective in the treatment of diabetes mellitus, and several studies have shown that flavonoids have beneficial effects on cancer, stress, heart disease and hypertension. Flavonoids are involved in insulin-sensitive tissues and many intracellular signaling pathways by regulating insulin secretion, carbohydrate digestion and glucose consumption. According to recent studies, by separating the flavonoids, i.e. 5-deoxyflavone (geraldone), Luteolin and Isookanin from the ethanolic extract of the plant *Albizia lebbeck* and molecular docking of these flavonoids on the alpha-glucosidase enzyme, those compounds could have anti alpha-glucosidase activities and
antidiabetic properties in vitro (Danesh et al., 2014). Increased antioxidant activity in aerial organs may be associated with increased phenolic and flavonoid compounds, and at higher concentrations of phenolic and flavonoid compounds, due to increased reactivity of hydroxyl agents, the possibility of hydrogen donation to free radicals and as a result, the extract’s inhibitory capacity may increase. Since phenolic and flavonoid compounds have antioxidant effects, it can be said that these compounds are the main cause of the antioxidant activity of the above-mentioned plants.

Conclusions

The results of this study indicate that the hexagonal extract of Ferula haussknechtii flower and Haplophyllum acutifolium has a significant inhibitory effect on alpha-glucosidase activity. Therefore, these results may be used in future studies to identify potential sources for the development of new drugs to prevent the progression of diabetes and to treat it. Hexane extract of both herbs showed high antioxidant activity, given that in recent years there has been a great deal of attention to natural antioxidants for health reasons. These plants may be used to access compounds with antioxidant properties.

References


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