Abstract. – Objectives: The Ferula genus (Umbelliferae) is a rich source of gum-resin and is much utilized in folklore medicine. This study is designed to examine antioxidant and anti-haemolytic activities of Ferula foetida regel flower, stem and leaf extracts.

Materials and Methods: 1,1-Diphenyl-2-picryl hydrazyl radical (DPPH), nitric oxide and H2O2 scavenging activities, Fe2+ chelating ability, reducing power and hemoglobin-induced linoleic acid peroxidation were used to evaluate antioxidant activities. Antihaemolytic activity was evaluated by H2O2 induced hemolysis in rat erythrocyte. Total phenolic compounds were determined as gallic acid equivalents and total flavonoid contents were calculated as quercetin equivalents from a calibration curve.

Results: The leaf aqueous-ethanol extract showed the highest activity in DPPH radical scavenging activity. All extracts showed weak nitric oxide scavenging activity. The stem extract had better activity in nitric oxide scavenging model than the other extracts (IC50 = 896.9 ± 21.9 µg ml−1), but it was not comparable to quercetin (p<0.001). The leaf extract exhibited better H2O2 scavenging and Fe2+ chelating activity than the other parts. The extracts exhibited good antioxidant activity in linoleic acid peroxidation test but were not comparable to vitamin C (p<0.001). Extracts showed weak reducing power activity. The stem extract showed better antihaemolytic activity than the flower and leaf. The flower extract had higher phenolic contents. The extracts exhibited different levels of antioxidant and antihaemolytic activities in all tested models.

Conclusions: This study showed remarkable antioxidant and antihemolytic activities in Ferula foetida. Biological effects may be attributed to the presence of phenols and flavonoids in the extract. It is very promising for further biochemical experiments.

Key Words: Antioxidant activity, Chelating activity, DPPH, Ferula foetida, Flavonoid, Nitric oxide scavenging.

Introduction

It is commonly accepted that under situations of oxidative stress, reactive oxygen species (ROS) such as superoxide, hydroxyl and peroxyl radicals are generated. The ROS play an important role related to degenerative or pathological processes such as aging, cancer, coronary heart disease, Alzheimer’s disease, neurodegenerative disorders, atherosclerosis, cataracts, and inflammation1,2. Minimizing oxidative damage may well be one of the most important approaches to the primary prevention of these aging-associated diseases ad health problems, since antioxidants terminate direct ROS attacks and radical-mediated oxidative reactions, and appear to be of primary importance in the prevention of these diseases and health problems1. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ or externally supplied through foods and/or supplements. Endogenous and exogenous antioxidants act as free radical scavengers by preventing and repairing damage caused by ROS and, therefore, can enhance the immune defense and lower the risk of cancer and degenerative diseases2,4. The use of traditional medicine is widespread and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs1. Consequently, the need to identify alternative natural and safe sources of food antioxidants arose and the search for natural antioxidants, especially of plant origin, has notably increased in recent years5,6. The Ferula genus (Umbelliferae) has been found to be a rich source of gum-resin7. This resin enjoys a reputation as a folklore medicine8. Sedative, carminative, anti-spasmodic digestive, expectorant, laxative, analgesic, anthelmintic, antiseptic and diuretic properties have been reported from the Ferula genus8.
It is also believed to have aphrodisiac and increasing sexual appetite\textsuperscript{10}. This genus presents interesting phytochemical features, such as the occurrence of sesquiterpenes and sesquiterpene coumarins\textsuperscript{8,11}. *Ferula foetida regel* is a perennial plant, which blooms once in its several years of life\textsuperscript{12,13}. It is native to central Asia, Afghanistan and Iran\textsuperscript{12}. Previously polysulfide derivatives\textsuperscript{14} and sesquiterpene coumarins\textsuperscript{15} were reported from *Ferula foetida*. *In vitro* fungi toxicity of the resin extract of *Ferula foetida* has been studied\textsuperscript{16}. We have recently reported good antioxidant activity from *Ferula assafoetida*\textsuperscript{9} and *Ferula gummosa*\textsuperscript{17,18}. The aim of this study was to determine the antioxidant and antihemolytic activities of the hydroalcoholic extract of *Ferula foetida regel* leaf, flower or stems in order to understand the usefulness of this plant as a medicinal plant.

### Materials and Methods

**Chemicals**

Trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydroxyl (DPPH), potassium ferricyanide and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) were purchased from Sigma Chemicals Co. (St Louis, MO, USA). Butylated hydroxyanisole (BHA), ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, ethylenediaminetetraacetic acid (EDTA) and ferric chloride were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade or purer.

**Plant Materials and Preparation of Freeze-Dried Extract**

*Ferula foetida regel* was collected from Gadouk area, central Elburz, Iran, in 2009. The sample was identified by Dr. Bahman Eslami (Assistant Professor of plant systematic, Islamic Azad University, Ghaemshahr Branch, Iran). Voucher specimens were deposited in the Herbarium of Sari Faculty of Pharmacy (No GRF 32-34). A known amount of each sample (250 g) was extracted at room temperature for 24 h by percolation with ethanol/water (600 ml, 70/30 v/v). The extract was then separated from the sample residue by filtration through Whatman No. 1 filter paper. This procedure was repeated thrice. The resulting extract was concentrated over a rotary vacuum until a crude extract was obtained, which was then freeze-dried for complete solvent removal.

**Determination of Total Phenolic Compounds and Flavonoid Content**

Total phenolic compound contents were determined by the Folin-Ciocalteau method\textsuperscript{19}. The extract samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagents for 5 min and 2.0 ml of 75 g l\textsuperscript{-1} sodium carbonate was then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoids were estimated as previously described\textsuperscript{20}. Briefly, 0.5 ml solution of each extracts in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (UV–Visible EZ201, Perkin Elmer, USA). Total flavonoid contents were calculated as quercetin from a calibration curve.

**Antioxidant activity**

**DPPH Radical-Scavenging Activity**

The stable 1, 1-diphenyl-2-picryl hydroxyl radical (DPPH) was used for determination of free radical scavenging activity of the extracts\textsuperscript{21}. Different concentrations of extracts were added, at an equal volume, to the methanol solution of DPPH (100 µM). After 15 min at room temperature, the absorbance was recorded at 517 nm (UV–Visible EZ201, Perkin Elmer, USA). The experiment was repeated three times. Vitamin C, BHA and quercetin were used as standard controls. IC\textsubscript{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

**Determination of Metal Chelating Activity**

The ability of the *Ferula foetida regel* extracts to chelate ferrous ions was estimated in our recently published paper\textsuperscript{22}. Briefly, different concentrations of each extract were added to a solution of 2 mM FeCl\textsubscript{2} (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixture was then shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solutions was measured spectrophotometrically at 562 nm (UV–Visible EZ201, Perkin Elmer, USA). The percentage inhibition of ferrozine-Fe\textsuperscript{2+} complex formation was
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Elmer, USA). Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

**Assay of Nitric Oxide-Scavenging Activity**

Sodium nitroprusside (10 mM), in phosphate-buffered saline (PBS), was mixed with different concentrations of extracts dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the extracts but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H₃PO₄ and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control.

**Scavenging of Hydrogen Peroxide**

The ability of the extracts to scavenge hydrogen peroxide was determined according to our recently published paper. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. All the extracts (0.1-3.2 mg ml⁻¹) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of hydrogen peroxide at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extracts and standard compounds was calculated as follows: % scavenged \( [\text{H}_2\text{O}_2] = \{(A_0 - A_1)/A_0\} \times 100 \), where \( A_0 \) was the absorbance of the control and \( A_1 \) was the absorbance in the presence of the sample of extract and standard.

**Reducing Power Determination**

The reducing power of extract was determined according to our recently published papers. 2.5 ml of extract (25-800 mg ml⁻¹) in water was mixed with phosphate buffer (2.5 ml, 0.2M, pH 6.6) and potassium ferricyanide \( [\text{K}_3\text{Fe(CN)}_6] \) (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm (UV–Visible EZ201, Perkin Elmer, USA). Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.
Statistical Analysis

Experimental results are expressed as means ± SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p<0.05$) and the means separated by Duncan’s multiple range tests. The EC50 values were calculated from linear regression analysis.

Results

Total phenol and flavonoids contents of extracts obtained from *Ferula foetida* leaf, flower, and stem are shown in Table I. The maximum of extractable polyphenol content was recorded in flower with 51.7 ± 2.3 mg gallic acid equivalent/g of extract, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.987$). In addition, maximum flavonoid contents were recorded in leaf extract with 20.9 ± 1.4 mg quercetin equivalent/g of extract, by reference to standard curve ($y = 0.0067x + 0.0132$, $r^2 = 0.999$). IC50 for DPPH radical-scavenging activity exists in Table 1. The IC50 values for vitamin C, quercetin and BHA were 5.05 ± 0.1, 5.28 ± 0.2 and 53.96 ± 3.1 mg ml⁻¹, respectively. Results of iron chelating capacity were presented in Table I. Among the extracts, leaf extract showed better activity than others with IC50 = 302.2 ± 13.6 µgml⁻¹. EDTA showed very powerful activity (IC50 = 18 ± 1.5 µgml⁻¹). In scavengers of nitric oxide, percentage of inhibition was increased with increasing concentration of the extracts but the activity was very weak (IC50 for stem extract was 896.9 ± 21.9 µgml⁻¹ vs. quercetin which was 20 ± 0.1 µgml⁻¹). The extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner. Results exist in Table 1. Leaf extract showed better activity than others (IC50 was 105.7 ± 4.7 mg ml⁻¹). The IC50 values for vitamin C and quercetin were 21.4 ± 1.1 and 52 ± 2.6 mg ml⁻¹, respectively. Figure 1 shows the dose response curves for the reducing powers of the extracts. It was found that the reducing powers of all extracts increased with the increase of their concentrations. There were no significant differences ($p>0.05$) among the extracts in reducing power. Their activities were not comparable with vitamin C ($p<0.001$). Tested extracts showed good activity in hemoglobin-induced linoleic acid system but there were significant differences between extracts and vitamin C ($p<0.01$) (Figure 2). The effects of extracts were tested and it was found that they did not show any harmful effects on erythrocytes. Results are shown in Table I. Stem extract showed better antihemolytic activity than other extracts (IC50 was 248.7 ± 16.2 µg ml⁻¹ vs. vitamin C which was 235 ± 9 µg ml⁻¹).

Discussion

Extracts showed high level of total phenol and flavonoid contents. Phenols and polyphenolic compounds, such as flavonoids, are widely found...
Table I. Phenol and flavonoid contents, antioxidant and antihemolytic activities of flowers, stems and leaves of *Ferula foetida* regel.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Antihemolytic activity (µg ml⁻¹)</th>
<th>Fe³⁺ chelating ability, IC₅₀ (µg ml⁻¹)</th>
<th>H₂O₂ scavenging activity, IC₅₀ (µg ml⁻¹)</th>
<th>Nitric oxide scavenging, IC₅₀ (mg ml⁻¹)</th>
<th>DPPH free radical scavenging, IC₅₀ (µg ml⁻¹)</th>
<th>Total flavonoid contents (mg g⁻¹)</th>
<th>Total phenol contents (mg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flower</td>
<td>260.4 ± 11.5</td>
<td>775.5 ± 21.4</td>
<td>193.8 ± 8.6</td>
<td>1.2 ± 0.04</td>
<td>471.6 ± 19.4</td>
<td>20.7 ± 0.9</td>
<td>51.7 ± 2.3</td>
</tr>
<tr>
<td>Leaf</td>
<td>253 ± 9.4</td>
<td>302.2 ± 13.5</td>
<td>105.7 ± 4.7</td>
<td>1.4 ± 0.03</td>
<td>192.5 ± 8.6</td>
<td>20.9 ± 1.4</td>
<td>49.4 ± 2.1</td>
</tr>
<tr>
<td>Stem</td>
<td>248.7 ± 16.2</td>
<td>619.6 ± 18.9</td>
<td>150.2 ± 5.2</td>
<td>0.89 ± 0.01</td>
<td>775.6 ± 26.3</td>
<td>9.9 ± 0.3</td>
<td>36.7 ± 1.3</td>
</tr>
</tbody>
</table>

IC₅₀ of BHA was 53.96 ± 3.1, vitamin C, 5.05 ± 0.1 and quercetin 5.28 ± 0.2, respectively. IC₅₀ of quercetin was 20 ± 0.1. IC₅₀ for vitamin C and quercetin were 21.4 ± 1.1 and 52 ± 2.6, respectively. EDTA used as control (IC₅₀ = 18 ± 1.5 µg ml⁻¹). IC₅₀ of vitamin C was 235 ± 9.
in food products, derived from plant sources, and they have been shown to possess significant antioxidant activities. DPPH is a stable nitrogen-centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers. Phenol and flavonoid contents of this plant may lead to its agreeable antioxidant activities. DPPH-scavenging activity. Iron chelators reduce iron-related complications in human and thereby improves quality of life and overall survival in some diseases such as thalassemia major or Alzheimer’s disease. Bivalent transition metal ions play an important role as catalysts of oxidative processes. These processes can be delayed by iron chelation and deactivation. The transition metal, iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular diseases. Thus, minimizing Fe²⁺ concentration affords protection against oxidative damage. Many researches focused on some natural products, especially flavonoids that possess direct influence on iron (III) ions level within tissues. Ferrozine can quantitatively form complexes with Fe²⁺. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The nicotinic acid assay is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of NO compete with oxygen, leading to reduced production of nitrite ions. Extracts did not show any in scavenging NO.

Scavenging of H₂O₂ by extract may be attributed to its phenolics, and other active components which can donate electrons to H₂O₂, thus neutralizing it to water. Although H₂O₂ itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H₂O₂ is very important throughout food systems. Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe³⁺ to Fe²⁺ by donating an electron. Amount of Fe²⁺ complex can be then be monitored by measuring the formation of Perl’s Prussian blue at 700 nm. Increasing absorbance indicates an increase in reductive ability. Reducing powers of extracts increased with the increase of their concentrations, but their activities were not comparable with vitamin C (p<0.001). Polyphenolic contents of all the sample extracts appear to function as good electron and hydrogen atom donors and, therefore, should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. Similar observation between the polyphenolic constituents in terms of dose dependent and reducing power activity have been reported for several plant extracts.

Erythrocytes are considered as prime targets for free radical attack, owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the O₂ transport associated with redox active hemoglobin molecules, which are potent promoters of reactive O₂ species. Specially linoleic acid and arachidonic acid are targets of lipid peroxidation. The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical. Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Tested extracts show good activity in hemoglobin-induced linoleic acid peroxidation test. Effect of extracts were tested and found that they did not show any harmful effects on erythrocytes.

Flavonoids interactions with cell membranes which generally serve as targets for lipid peroxidation (LP), constitute an important area of research. Various model membrane systems like LDL and red blood cells (RBC) membrane comprising physiologically important membrane protein components offer a physiologically relevant and a relatively simple system for studying LP. RBC has been chosen as an in vitro model to study the oxidant/antioxidant interaction since its membrane is rich in polyunsaturated fatty acids, which are extremely susceptible to peroxidation. During recent years, a few interesting studies have been reported, indicating the protective effects of some plants extracts against oxidative damage in intact RBC membranes.
free radical damage and its inhibition by antioxidants but only few studies have been performed with erythrocytes in whole blood. In this study, we used a biological test based on free radical-induced erythrocytes lysis in rat blood. Lipid oxidation of rat blood erythrocyte membrane mediated by hydrogen peroxide induces membrane damage and subsequently hemolysis. Stem extract showed better antihemolytic activity than other extracts (IC₅₀ was 248.7± 16.2 µg ml⁻¹ vs. vitamin C which was 235 ± 9 µg ml⁻¹). The antihemolytic activity of flavonoids has been previously reported and activity of the extract maybe results in high flavonoid content⁹,3⁸.

Conclusions

Our study improved remarkable antioxidant and antihemolytic activities in hydroalcoholic extract of Ferula foetida regel Boiss flower, stem and leaf. These effects maybe result of their high phenol and flavonoids contents. It is therefore very promising for further biochemical experiments, which will be focused on evaluating the mechanism of this activity.

References


3) TEPE B, SOKMEN A. Screening of the antioxidative properties and total phenolic contents of three endemic Tanacetum subspecies from Turkish flora. Bioresource Technol 2007; 98: 3076-3079.


22) EBRABHIMZADEH MA, NABAVI SM, NABAVI SF. Correlation between the in vitro iron chelating activity


