Original Article

Antioxidant Activity of the *Ferula gummosa* Boiss.'s Aerial Parts: Measurements Based on Different Assay Methods

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Abstract

Introduction: Measuring of natural antioxidants power is important in the food industry. *Ferula gummosa* Boiss. plant, locally called Barijeh, is a member of genus *Ferula* belonging to the Apiaceae family. To introduce endemic natural antioxidants, antioxidant capacity of alcoholic and hydroalcoholic extracts of aerial parts of *F. gummosa* Boiss. was investigated. **Objective:** The primary objective of this study was to compare the antioxidant levels and activities between flower and leaf extracts of *Ferula gummosa* Boiss. plant by different assay methods. **Method:** The antioxidant activity of flower and leaf extracts of *F. gummosa* Boiss. was assessed usingferric-reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and oxygen radical absorbance capacity (ORAC) assay. In addition, phenolic content of the extracts was measured byFolin-Ciocalteu (FC) method. **Results:** Ferric reducing antioxidant power assay showed that leaf extract has more antioxidant activity compared to flower extract. DPPH assay had similar results. A slow kinetic behavior was found for methanol extracts of both tissues (EC_{50} of 0.21 mg/mL and 0.25 mg/mL for leaf and flower methanol extracts, respectively) which was estimated by kinetic mode of DPPH assay. The ORAC assay showed higher values for methanolic extracts compared to ethanolic extracts. Except for ORAC assay, a significant positive correlation was found between antioxidant data of ferric-reducing antioxidant power, DPPH and Folin-Ciocalteu assays. **Conclusion:** These findings suggest that high antiradical potential and reducing power of the alcoholic and hydroalcoholic extracts of the aerial parts of *F. gummosa* Boiss. correspond to a high phenolic content in these plant parts. The high antioxidant activity of the *F. gummosa* Boiss. could propound the hydroalcoholic extracts of this plant as a therapeutic agent to prevent and treat diseases due to free radical imbalance in the body.

Keywords: *Antioxidant activity, DPPH, ferric-reducing antioxidant power, Ferula gummosa Boiss., oxygen radical absorbance capacity*

Introduction

Reactive oxygen species are side products of biochemical reactions, mainly in electron transfer chain reactions in mitochondria.[1] Their excess in the body can damage vital macromolecules including proteins, lipids, and DNA, resulting in oxidative stress. Oxidative stress means that there are more oxidants than antioxidants.[2,3] It can lead to cardiovascular diseases[4,5] and cancer.[6]

Epidemiological studies have shown that if more antioxidants, especially phenolic compounds, are consumed, there will be decreased mortality because of cardiovascular diseases.[7] Hence, food industry has used synthetic antioxidants as food additives to extend shelf life and inhibit lipid rancidity.[8,9] However, demand for natural antioxidants has increased due to concerns about synthetic antioxidants' safety.[10-12] Therefore, there has been increasing efforts to identify new antioxidants from natural resources. These natural antioxidants can design nutraceuticals that prevent oxidative damages in the body.

Ferula gummosa Boiss. plant, locally called Barijeh in western and northern mountains of Iran (contemporary Persia), is a member of genus *Ferula* belonging to the *Apiaceae* family.[13] It grows in spring on 1900–3200 m altitude above the sea level. "Barijeh" is the name given to this plant by Iran's nomads. Its resin has conventionally been used for treating diarrhea. The nomads believe that it is an effective antidiarrheal herbal medicine.[14] In traditional Persian medicine, the aerial parts' gum of this plant has been used for stomach pain, chorea, epilepsy and wound healing.[15]

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There are some reports regarding the medicinal effects of *F. gummosa* Boiss. For example, it has been shown that the hydroalcoholic extract of the aerial parts of the plant has antinociceptive activity.[16] This curative effect has also been reported for seed and root acetone extract of the plant.[17] Furthermore, a methanol-chloroform (1:1) extract of this plant and its fractions has alleviated the morphine withdrawal syndrome induced by naloxone.[18] Moreover, the anticonvulsant potential of an essential oil,[19] the antibacterial activity of its seed,[20] and the anti-inflammatory activity of its seed and root[17] have been reported.

In this study, three assay methods were used to measure the antioxidant contents of the flower and leaf extracts of *F. gummosa* Boiss. plant: ferric-reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and oxygen radical absorbance capacity (ORAC) assays. In addition, Folin–Ciocalteu (FC) reagent was used to determine the total phenolic content (TPC) of the extracts. Based on the reaction mechanism, FRAP, DPPH, and FC assays are categorized as electron-transfer methods and ORAC assay as a hydrogen-transfer method.[21]

Materials and Methods

Plant material

F. gummosa Boiss. was collected from Damavand Mountains in the north of Iran and identified by Dr. Seyed Mohammad Masoumi, Assistance Professor of Botany, Razi University, Kermanshah, Iran. A voucher herbarium specimen (No. 576) was deposited in the Herbarium of Biology Department Razi University.

Chemicals and reagents

The chemicals 2,2'-azobis (2-amidinopropane), dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8 -tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich GmbH (Munich, Germany). 2,4,6-tripyridyl-s-triazine (TPTZ), fluorescein, 2,2-diphenyl-1-picrylhydrazyl (DPPH), iron (III) chloride hexahydrate ($FeCl_3.6H_2O$), sodium tungstate, sodium molybdate, sodium carbonate (Na_2CO_3), sodium acetate, sodium sulfate, and all of the used solvents were purchased from Merck (Darmstadt, Germany). All solvents and reagents were of analytical grade.

Sample preparation and extraction procedure

Aerial parts of *F. gummosa* Boiss. plant were dried at room temperature 20°C–25°C and darkness for 7 days. The dried flowers and leaves were grounded in a blender. About 50 g of the grounded powders was placed separately in two round bottom bottles. They were mixed with petroleum ether (each three times) until discoloration. Afterward, the two almost resin-free suspensions were centrifuged at 2700 g for 10 min. The obtained pellets were remixed with 500 mL ethanol and stirred for 4 days. After centrifugation at 2700 g for 15 min, the supernatants were evaporated under vacuum to dry out. The resultant powders were flower or leaf ethanol extracts. They were stored at 4°C until analysis. Subsequently, flower or leaf ethanol extract powders were re-dissolved in methanol (50%):chloroform (1:1 v/v) mixtures separately. The methanol phases (flower or leaf methanol extracts) were evaporated under vacuum to dry out.

Total phenolic content assay

The TPCs in flower's ethanol and methanol extracts and leaf's ethanol and methanol extracts were determined by FC assay[22,23] with some modifications. Appropriate extract dilutions (50 µl) were added to FC reagent (50 µl, 0.2 N) and double distilled water (800 µl). After 5 min, Na_2CO_3 (100 µl, 0.5 M) was added. The mixtures were incubated for 1 h at room temperature, and the resulting light blue color solution absorbance was measured at 725 nm using a Cary-100 Bio-spectrophotometer (Varian, California, USA). Trolox was used as the standard antioxidant. The standard curve was linear between 0 and 200 µM Trolox. Results were presented as µmol of Trolox equivalent per gram of plant extracts' dry mass ($\mu mol_{TE}/g_{DM}$).

Ferric-reducing antioxidant power assay

The FRAP assay was done according to Benzie and Strain's method[24,25] with some modifications. The stock solutions included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The FRAP solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3.6H_2O$ solution. It was warmed up to 37°C before use. Flower ethanol and methanol extracts and leaf ethanol and methanol extracts (10 µl) were allowed to react with 990 µl of the FRAP solution for 30 min in the dark condition. The colored products were read at 595 nm using a Cary-100 Bio-spectrophotometer. Trolox was used as a standard antioxidant to express the extracts' ferric ion-reducing power. The standard curve was linear between 5 and 20 µM Trolox. Results were expressed in $\mu mol_{TE}/g_{DM}$.

DPPH assay

The DPPH assay was done in kinetic and nonkinetic modes according to Miliauskas *et al.*[26] and Bran-Williams *et al.*[27] methods with some modifications. In nonkinetic mode, 50 µL of all extracts was mixed with 950 µL of the DPPH solution (6×10^{-5} M DPPH in methanol) for 24 h in the dark. The bleached products' absorbance was then recorded at 515 nm using a Cary-100 Bio-spectrophotometer. Trolox was used as a standard antioxidant to express extracts' antiradical power. The standard curve was linear between 5 and 50 µM Trolox. Results were expressed in $\mu mol_{TE}/g_{DM}$.

In kinetic mode, 50 µL of different concentrations (50, 100, 200, and 300 µg/mL in methanol) of the flower

and leaf methanol extracts was mixed with 950 μL of DPPH solution and the decrease in absorbance was recorded at 515 nm with 30 s time intervals until 3 h and thereafter at 2 h time intervals until the reaction reached a steady state. For each tested flower and leaf methanol extracts concentration, the reaction kinetics was plotted by the equation:

$$\%DPPH = 100 \times DPPH_{rem} / DPPH_{t=0}$$

Here, $DDPH_{rem}$ is the remaining DPPH concentration at different times and $DPPH_{t = 10}$ is amount of DPPH at initial time. From these graphs, the remaining DPPH percentage at the steady state for each concentration of flower and leaf methanol extracts was determined. These values were plotted versus flower and leaf methanol extracts concentrations to calculate EC_{50} (the amount of antioxidant necessary to decline the initial DPPH concentration by 50%), using the exponential equation:

$$y = a \times \exp(-x / t_1) + y_0$$

where a is the slope and y_0 is the intercept.

Oxygen radical absorbance capacity-fluorescein assay

The ORAC assay was done according to the procedure described by Ou *et al.* and da Silva *et al.*[28,29] with some modifications. The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 500 μl. The flower or leaf ethanol extracts, flower and leaf methanol extracts (200 μl), and fluorescein (200 μl, 78 nM, final concentration) solutions were put in the fluorescence cuvette. The mixture was pre-incubated for 10 min at 37°C. AAPH solution (100 μl, 221 μM, final concentration) was added and the cuvette was immediately put in the fluorescence spectrophotometer (Cary Eclipse, Varian, California, USA) spectrofluorimeter with jacketed cell holder in which temperature was controlled by an external thermostated water circulation system. Fluorescence was recorded at 1-min intervals until it reached less than 5% of initial intensity (excitation wavelength 485 nm, emission wavelength 535 nm). Phosphate buffer was used for blank (fluorescein + AAPH) instead of the antioxidant solution. Different concentrations of Trolox were used as an antioxidant for standard solutions. Antioxidant curves (fluorescence versus time) corresponded to the same assay by multiplying original data by the factor $fluorescence_{blank,\ t\ =\ 0}$/$fluorescence_{sample,\ t\ =\ 0}$. From the normalized curves, the area under the fluorescence decay curve (AUC) was calculated as:

$$AUC = 1 + \frac{f_1}{f_0} + \frac{f_2}{f_0} + \frac{f_3}{f_0} + \ldots + \frac{f_n}{f_0}$$

In this formula, f_0 is the initial fluorescence read at 0 min and f_i is the fluorescence reading at time *i*. The net AUC corresponding to a sample was calculated by subtracting the AUC corresponding to the blank. Regression equations between net AUC and antioxidant concentration were calculated for all the extracted samples. ORAC-fluorescein values were calculated as

$$ORAC_{value} = C_{Trolox} \times [(AUC_{sample} - AUC_{blank})] / [(AUC_{Trolox} - AUC_{blank})]$$

where C_{Trolox} is molarity of Trolox. Final results were expressed in μmol of Trolox equivalent per gram of dry mass of the extracts ($\mu mol_{TE}/g_{DM}$).

Results and Discussion

In our study, a blue-colored solution was produced when the aerial parts extracts reacted with FC reagent and Na_2CO_3. This shows the presence of phenolic compounds in all the extracts. The TPC values of the ethanolic and methanolic extracts from the flower and leaf of *F. gummosa* Boiss. have been tabulated in Table 1. The TPC values ranged from 0.4 to 0.9 $\mu mol_{TE}/g_{DM}$. The highest phenolic content was obtained using methanolic extraction method. Thus, protocols using methanol (50%):chloroform mixture would be the preferred method to concentrate phenolic substances of high polarity.

Ferric ion-reducing capacities of the *F. gummosa* Boiss. extracts are listed in Table 1. The trend of the reducing power was similar to that of the TPC measured by FC assay. Again, the leaf methanolic extract with FRA*P* value of 120 $\mu mol_{TE}/g_{DM}$ had the highest reducing activity followed by leaf ethanol extract, flower methanol extract, and flower ethanol extract. Compared with vegetables such as *Quercus robur* (15.92 ± 0.17), *Cinnamomum zeylanicum* (6.48 ± 0.15), *Eugenia caryophyllus clovis* (7 ± 0.13), *Styrax benzoin* (3.08 ± 0.07), *Eucalyptus globulus* (4.66 ± 0.06), *Matricaria recutita* (0.12 ± 0.01),[30] *Emex spinosus* (2.21 ± 0.001), and *Asphodelus tenuifolius* (0.44 ± 0.002),[31] *F. gummosa* Boiss. extract has a significantly more ferric reducing potential. However, the solvents used to extract antioxidant compounds in the studies by Dudonne *et al.* and Al-Laitif *et al.* were different from ours.

The DPPH assay is a technically simple and reproducible method for evaluating radical scavenging capacities of plant extracts, fruits, olive oil, and wines.[27] It has kinetic and nonkinetic modes. Depending on reacting rate to the steady state, there are three types of kinetic reactions: rapid (<1 min), intermediate (5–30 min), and slow (>1 h).[27] DPPH radical scavenging activities of the flower's or leaf's ethanol extracts and the flower's and leaf's methanol extracts were in the range of 105–202 $\mu mol_{TE}/g_{DM}$s [Table 1] when calculated nonkinetically. Compared with vegetables such as *Aizoon canariense* (66.56 ± 2) and *A. tenuifolius* (20.93 ± 0.09)[31] *F. gummosa* Boiss. extract has a significantly more radical scavenging capacity. Interestingly, similar trends were observed in FRAP, DPPH, and TPC values (that is leaf methanol extract > leaf ethanol extract > flower methanol extract > flower ethanol extract). To evaluate antiradical behaviors of flower and leaf's methanol extracts kinetically, the time evolutions of remaining DPPH for each concentration of the extracts were plotted [Figure 1].

Table 1: Antioxidant activity of different extracts from *Ferula gummosa* Boiss.

Extract type	Total phenolic content value ($\mu mol_{TE}/g_{DM}$±SD)	FRAP value ($\mu mol_{TE}/g_{DM}$±SD)	DPPH value ($\mu mol_{TE}/g_{DM}$±SD)
Leaf ethanol	0.7±0.04	99±7.5	140±12.6
Flower ethanol	0.4±0.001	82.7±2.5	105.649±7.76
Leaf methanol	0.9±0.03	122.4±3.6	201.9±6.835
Flower methanol	0.6±0.01	92.5±8.9	132.5±3.8

Units of the values are in $\mu mol_{TE}/g_{DM}$ (μmol of trolox equivalent per gram of dry mass of plant extract). Data are expressed as the mean of triplicate±SD. SD: Standard deviation

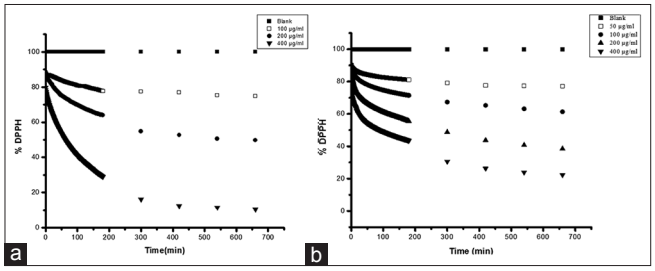

Figure 1: DPPH kinetic assay. Kinetic curves of methanolic DPPH solution with increasing concentrations of (a) leaf extract and (b) flower extract from *Ferula gummosa* Boiss. DPPH scavenging capacity of leaf extract is higher than flower extract in all applied concentrations. blank, 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml

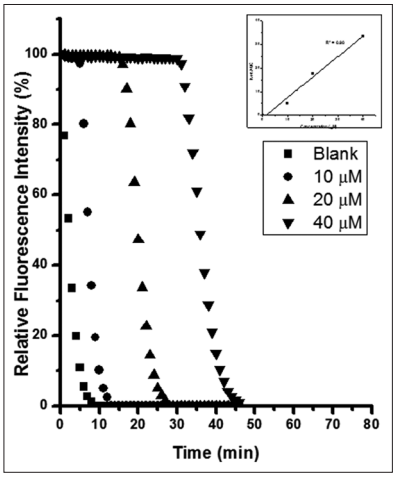

Figure 2: Oxygen radical absorbance capacity assay for Trolox as a standard antioxidant. Oxygen radical absorbance capacity assay for Trolox at the indicated initial concentrations, illustrating time-dependent loss of fluorescence of fluorescein's solutions at presence of 2,2'-azobis (2-amidinopropane), dihydrochloride as a biologically relevant radical. With increasing concentrations of Trolox, the time needed for quenching of fluorescein and, consequently, net area under the fluorescence decay curve is increased (inset). Blank, 10 μM, 20 μM, 40 μM

Antioxidant compounds available in the flower and leaf's methanol extracts had slow kinetic behaviors in our study. Furthermore, the free radical scavenging activities of flower and leaf methanol extracts represented by EC_{50} were 0.21 and 0.25 mg/mL, respectively. These values were in the range of EC_{50} of known antioxidants such as curcumin, butylated hydroxytoluene, thymol, and carvacrol, having EC_{50} values of 0.0078, 0.02, 0.16, and 0.25 mg/ml, respectively.[32]

The ORAC assay is based on hydrogen atom transfer mechanism and employs a biologically relevant radical,[21,30-32] and it is the only method quantifying both inhibition time and degree of inhibition for an antioxidant. Moreover, the ORAC assay is the only method that is able to evaluate the kinetic behaviors of antioxidants by calculation of area under the decay curves of fluorescein.[33] These discrepancies might explain the low correlation coefficients observed between the ORAC assay and other methods that are expressed in following section. Figure 2 illustrates fluorescein decay curves for Trolox as a standard antioxidant over a concentration range of 0–40 μM. There was a positive correlation between the concentration of Trolox and required time for the decay in fluorescence intensity of fluorescein. The net area under the curve (net AUC) was plotted as a function of Trolox concentrations. Similarly, fluorescence decay curves and corresponding net AUCs were plotted for the flower or leaf's ethanol extracts and the flower and leaf's methanol extracts of *F. gummosa* Boiss. [Figure 3a-d]. From the ORAC values of the extracts [Table 2], the leaf methanol extract showed the highest value (3078 $\mu mol_{TE}/g_{DM}$) and the flower ethanol extract represented the least value (2152 $\mu mol_{TE}/g_{DM}$). Compared to several known extracts, the flower and leaf extracts of *F. gummosa* Boiss. show higher antioxidant capacities. For example, the extracts from black tea and blueberry plant leaves have the ORAC values of 1629 and 2792 $\mu mol_{TE}/g_{DM}$, respectively.[34] These values were 2152–3078 $\mu mol_{TE}/g_{DM}$ for *F. gummosa* Boiss. extracts [Table 2]. However, it is noticeable that the methods and the solvents used by Atala *et al.* had been different from ours. *F. gummosa* Boiss. extracts' ORAC values are much lower than that of grape skin which is 15675 $\mu mol_{TE}/g_{DM}$. This high antioxidant activity is also observed in citrus families[35] and different parts of the orangery plant.[36,37]

To draw out relationships between the methods used in this study, correlations among the methods were calculated by regression analysis on results of various methods [Table 3]. A significant positive correlation was found between FRAP and DPPH assays (R = 0.99). Further, the results of DPPH and FRAP assays were significantly correlated with the TPC concentration based on FC method with 0.91 and 0.94 correlation coefficients, respectively. These high correlation coefficients indicate that there are good relationships between phenolic compound concentration in

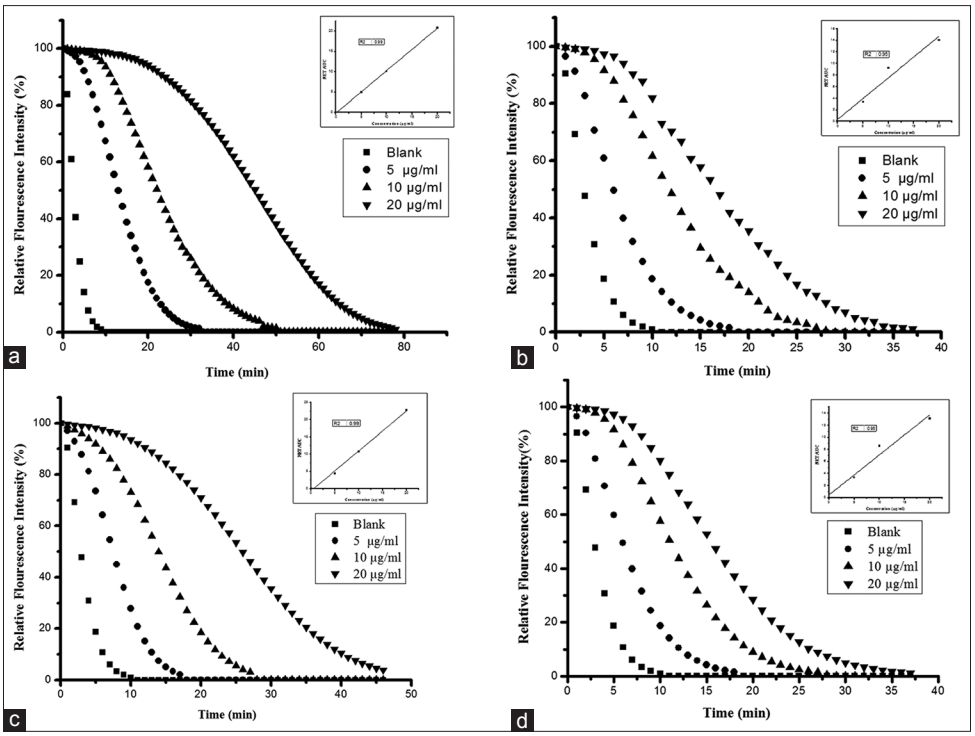

Figure 3: Oxygen radical absorbance capacity-fluorescein curves at presence of *Ferula gummosa* Boiss. extracts. Fluorescence decay curves of fluorescein at presence of (a) ethanolic leaf's extract, (b) ethanolic flower's extract, (c) methanolic leaf's extract, and (d) methanolic flower's extract of *Ferula gummosa* Boiss. Based on the net area under the fluorescence decay curve profiles (insets on the figure), methanolic flower's extract has maximum delaying effect on fluorescein consumption induced by 2,2'-azobis (2-amidinopropane), dihydrochloride, and methanolic leaf's extract, ethanolic leaf's extract, and ethanolic flower's extract occupy next ranks, respectively. Blank, 5 µg/ml, 10 µg/ml, 20 µg/ml

Table 2: Comparison of oxygen radical absorbance capacity-fluorescein values in alcoholic extracts of *Ferula gummosa* Boiss. with some of known extracts in literature

Extract type	Extraction solvents (solvent ratio)	ORAC value $\mu mol_{TE}/g_{DM}$
Ferula gummosa Boiss.		
Leaf methanol	Methanol (50%): Chloroform (1:1)	3078 (this study)
Flower methanol	Methanol (50%): Chloroform (1:1)	2276 (this study)
Leaf ethanol	Ethanol (100%)	2948 (this study)
Flower ethanol	Ethanol (100%)	2152 (this study)
Black tea leaf	Acetone: water (4:1)	1629[34]
Blueberry	Acetone: water (4:1)	2792[34]
Grape skin	Acetone: water (4:1)	15675[34]
Matricaria recutita (flower)	Water	588[31]
Lavandula hybrida Grosso. (flower)	Water	1181[31]
Actinidia chinensis (flower)	Water	877[31]
Cistus ladaniferus (leaf)	Water	1410[31]

Units of the values are in $\mu mol_{TE}/g_{DM}$ (µmol of trolox equivalent per gram of dry mass of plant extract). Data are expressed as the mean of triplicate±SD. SD: Standard deviation. ORAC: Oxygen radical absorbance capacity

F. gummosa Boiss. extracts and their antiradical capacity as well as FRAP. In other words, higher phenolic content can lead to higher antioxidant potential. Antioxidant potential of phenolic compounds is related to a number of hydroxyl groups and other substitutions on their aromatic rings.[38] The lowest correlations were found between ORAC and FRAP (R = 0.014), ORAC and DPPH (R = 0.002), and ORAC and TPC (R = 0.07) assays. Since the antioxidant properties of phenolic compounds depend on their structure, the significant correlation between the electron-transfer-based methods and TPC content was expectable. Having hydroxyl groups attached to aromatic rings, phenolic compounds can quench free radicals by a resonance-stabilized mechanism.[23,32]

As it is clear, the mechanism of electron-transfer methods such as FRAP, DPPH, and FC assay (which are based on the reduction of persistent radicals or of inorganic oxidizing species) is different from hydrogen-transfer methods such

Table 3: Correlation coefficient between electron-based mechanism methods and hydrogen-based mechanism method

Assay methods	FRAP	DPPH	TPC	ORAC
FRAP	1			
DPPH	0.99	1		
TPC	0.94	0.91	1	
ORAC	0.014	0.002	0.07	1

FRAP: Ferric-reducing antioxidant power, DPPH: 2,2-diphenyl-1-picrylhydrazyl, TPC: Total phenolic content, ORAC: Oxygen radical absorbance capacity

as ORAC (which is based on the competitive bleaching of a probe).[39] Hence, we were convinced that why the results of the first three methods showed close relationship, and there was weak correlation with ORAC assay which is a hydrogen-transfer method. It has been reported that *Ferula* is a genus rich in coumarins, particularly sesquiterpene coumarins,[40] and our results also confirmed that *F. gummosa* Boiss. has a high phenolic content. Therefore, *F. gummosa* is a promising source that shows the high potency of omitting different kinds of free radicals. However, more investigations are required to analyze phenolic contents of the solvent-based extracts of *F. gummosa* precisely.

It should be noted that to use of plant extracts in food industry, cytotoxicity of the extracts must be evaluated scrupulosity. In previous studies, several authors mentioned about cytotoxicity of different parts of *F. gummosa* Boiss., which we would rely on their result. For example, as a preliminary work, Gharaei *et al.* have shown that the ethanolic extracts of flower and leaf of *F. gummosa* Boiss. have cytotoxic effects on AGS cell line at concentrations of 50 and 60 μg/mL.[41] However, use of the solvent-based extracts of *F. gummosa* Boiss. as food additive requires more experimental studies to be done in future.

Conclusion

The antioxidant activity of the aerial parts of *F. gummosa* Boiss. was measured by FC, FRAP, DPPH, and ORAC assays. The first three methods had a high correlation in measuring antioxidant activity. Our study depicts that the alcoholic and hydroalcoholic extracts of aerial parts of *F. gummosa* Boiss. have remarkable antioxidant activity that may be as a result of their high phenol contents. The high antioxidant activity of the *F. gummosa* Boiss. could bring up the hydroalcoholic extracts of this plant as a therapeutic agent to prevent and treat diseases due to free radical imbalance in the body, such as Alzheimer and cancer . It is very promising to characterize the active compounds within these plant extracts that may be useful to introduce new nutraceutical agents and also focuses on identifying the mechanism of these activities.

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Conflicts of interest

There are no conflicts of interest.

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