Research Paper

Different Antioxidant Activity Measurements of the Aerial Parts of *Ferulago angulate*, Traditional Food Additives in Iran

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In this study ferric reducing antioxidant power assay, 2,2-diphenyl-1-picrylhydrazyl and oxygen radical absorbance capacity assays were employed to measure the antioxidant activity of flower and leaf extracts of *Ferulago angulata*. Ferric reducing antioxidant power assay of the extracts showed that the antioxidant activities of leaf extracts were higher than that of flower extracts. 2,2-diphenyl-1-picrylhydrazyl values showed similar trend to ferric reducing antioxidant power assay. A slow kinetic behaviour was found within methanol extracts of both plant parts (EC_{50} of 0.071 mg/ml and 0.12 mg/ml for leaf methanol extract and flower methanol extract, respectively), estimated by kinetic mode of 2,2-diphenyl-1-picrylhydrazyl assay. The oxygen radical absorbance capacity assay indicated higher values for methanol extracts of plant parts compared to that of ethanol extracts. Except for oxygen radical absorbance capacity assay, a significant positive correlation was found between ferric reducing antioxidant power, 2,2-diphenyl-1-picrylhydrazyl and Folin-Ciocalteu assays, suggesting that phenolic compounds have an important role in reducing power and antiradical properties of the extracts.

Key words: Antioxidant activity, DPPH, *Ferulago angulate*, FRAP, ORAC

Reactive oxygen species (ROS) are side products of numerous biochemical reactions; particularly in electron transfer chain reactions in mitochondria[1]. Excessive ROS in the body can lead to accumulative damages in vital macromolecules including proteins, lipids and DNA, resulting in oxidative stress. Oxidative stress, imbalance between oxidants and antioxidants in favor of the oxidants[2,3] has been considered as a cause of cardiovascular diseases[4,5] and cancer[6]. Epidemiological studies have shown an inverse relationship between consumption of antioxidants, especially phenolic compounds, and mortality from cardiovascular disease[7]. Owing to these statistical facts, the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) has prevailed as food additives in the food industry to extend shelf life and to inhibit lipid rancidity[8,9]. However, need for natural antioxidants was augmented due to concerns about the safety of synthetic antioxidants[10-12]. Therefore, efforts to identify new antioxidants from natural sources have being increased. These natural antioxidants have potential to design nutraceuticals, which can assist to prevent oxidative damages in the body.

In an effort to introduce endemic natural antioxidants, antioxidant capacity of alcoholic and hydroalcoholic extracts of aerial parts of *Ferulago angulate* were investigated. The aerial parts of this plant have traditionally added to different food products such as oil ghee to prevent rancidity and to impart a pleasant odour. *F. angulata*, locally called *Chavir* in west of Iran, is a member of genus *Ferulago* belonging to the Apiaceae family[13]. This plant is grown in the spring at an altitude of 1900-3200 m above sea level.

To measure antioxidant activities, three assays were used, ferric-reducing antioxidant power (FRAP) assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and oxygen radical absorbance capacity (ORAC) assay.

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Additionally, the Folin-Ciocalteu (FC) method was used to determine the total phenolic content (TPC) of the extracts. On the basis of mechanism of reaction, FRAP, DPPH and FC assays are categorized as electron-transfer methods, while ORAC assay is classified as a hydrogen-transfer method[14]. In addition, to determine the contribution of phenolic compounds to the antioxidant capacities of the extracts, correlations were evaluated among the applied methods by correlation coefficients analysis.

MATERIALS AND METHODS

F. angulata Boiss. was collected from the Dalani mountainous region in west of Iran during April and the so collected material was authenticated in the Department of Botany, Razi University, Kermanshah, Iran. A voucher herbarium specimen (No: 580) was deposited in the herbarium of the Agriculture Department of Razi University (HARU). 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 (FL), 2,2-diphenyl-1-picrylhydrazyl (DPPH), iron(III) chloride hexahydrate ($FeCl_3.6H_2O$), sodium tungstate ($Na_2WO_4.2H_2O$), sodium molybdate ($Na_2MoO_4.2H_2O$), sodium carbonate (Na_2CO_3), sodium acetate ($C_2H_3NaO_2$), sodium sulphate ($Na_2SO_4 \cdot 10H_2O$) and all of the solvents used in the present work were purchased from Merck, Darmstadt Germany. All solvents and reagents were of analytical grade.

Sample preparation and extraction procedure:

After harvesting, aerial parts of *F. angulata* were dried at room temperature 20-25° and darkness for 7 d. The dried flowers and leaves were ground in a blender and 50 g of them were put in a Soxhlet apparatus separately and were mixed with petroleum ether (~3 times) until discoloration. Afterwards, the two almost resin-free suspensions were centrifuged at 2700 g for 10 min, the pellets obtained were re-mixed with 500 ml ethanol and were stirred for 4 d. After centrifugation at 2700 g for 15 min, the supernatants were evaporated under vacuum to dryness and the resultant powders (henceforth referred to as ethanol extract of the flower (EF) or the leaf (EL) stored at 4° until analysis. The yield of dried flower and leaf extracts was about 7% (w/w) of the starting dried flower and leaf. Subsequently, the EF and EL powders were re-dissolved in methanol (50%):chloroform (1:1 v/v) mixture separately, and the methanol phases henceforth referred to as methanol extract of the flower (MF) or the leaf (ML) were evaporated under vacuum to dryness.

TPC assay:

The TPC in flower (EF and MF) and leaf (EL and ML) extracts was determined according to the FC method[15,16] with some modifications. Appropriate dilutions of the extracts (50 µl) were added to FC reagent (50 µl, 0.2 N) and double distilled water (800 µl). After 5 min, sodium carbonate (100 µl, 0.5 M) was added. The mixtures were incubated for 1 h at room temperature and the absorbance of the resulting light blue color solution was measured at 725 nm using a Cary-100 Bio spectrophotometer (Varian, Palo Alto, 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 dry mass of plant extracts ($\mu mol_{TE}/g_{DM}$).

FRAP assay:

The FRAP assay was performed according to Benzie and Strain[17,18] 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 fresh working solution (FRAP solution) was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml $FeCl_3.6H_2O$ solution and then warmed up to 37° before use. Flower (EF and MF) and leaf (EL and ML) extracts (10 µl) were allowed to react with 990 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored products were then taken at 595 nm using a Cary-100 Bio spectrophotometer. To express ferric ion reducing power of the extracts, Trolox was used as a standard antioxidant. 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 non-kinetic modes according to the method of Miliauskas *et al*.[19] and of Brand-Williams *et al*.[20] with some modifications. In non-kinetic mode, 50 µl of the plant extracts (EF, EL, MF and ML) were mixed with 950 µl of the DPPH solution (6×10^{-5} M DPPH in methanol) for 24 h in the dark. The absorbance of the bleached products was then recorded at 515 nm using a Cary-100 Bio spectrophotometer. To express antiradical power of the extracts, Trolox was used as

a standard antioxidant. 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, 300 μg/ml in methanol) of the MF and ML were mixed with 950 μl of DPPH solution and the decrease in absorbance was recorded at 515 nm at time intervals of 30 s until 3 h and thereafter at time intervals of 2 h until the reaction reached a steady state. For each MF and ML concentration tested, the reaction kinetics were plotted by use of the Eqn., $\%DPPH=100\times[DPPH]_{rem}/[DPPH]_{t=0}$, where, $[DPPH]_{rem}$ is remaining DPPH concentration at different times and $[DPPH]_{t=0}$ is amount of DPPH at initial time. From these graphs, the percentage of DPPH remaining at the steady state for each concentration of MF or ML was determined and these values were plotted versus ML or MF concentrations to calculate EC_{50} (the amount of antioxidant necessary to decline the initial DPPH concentration by 50%), using the exponential model: $y=a\times\exp(-x)/t_1)+y_0$, where, a is the slope and y_0 is the intercept.

ORAC-FL assay:

The ORAC assay was done according to the procedure described by Ou *et al*.[21,22] with some modifications. The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 500 μl. Samples (EF, EL, MF and ML, 200 μl) and FL (200 μl; 78 nM, final concentration) solutions were put in the florescence cuvette. The mixture was pre-incubated for 10 min at 37°. AAPH solution (100 μl; 221 μM, final concentration) was added and the cuvette was immediately put in the fluorescence spectrophotometer (Cary Eclipse, Varian, Palo Alto, 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). For blank (FL+AAPH), phosphate buffer was used instead of the antioxidant solution, and for standard solutions, different concentrations of Trolox were used as an antioxidant. Antioxidant curves (fluorescence versus time) were normalized to the curve of the blank 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: $AUC=1+f_1/f_0+f_2/f_0+f_3/f_0+\cdots+f_n/f_0$, where, f_0 is the initial fluorescence read at 0 min and f_1 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 samples. ORAC-FL 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

The TPC values of the ethanol and methanol extracts of flower (EF, MF) and leaf (EL, ML) ranged from 0.4 to 1 $\mu mol_{TE}/g_{DM}$ (Table 1). The highest phenolic content obtained using ethanolic extraction followed by methanolic extraction. Thus, protocols using methanol (50%):chloroform mixture would be the choice method to concentrate phenolic substances of high polarity. Antioxidant properties of phenolic compounds are directly depended on their structure. Having hydroxyl groups attached to aromatic rings, phenolic compounds are potentially able to quench free radicals by a resonance-stabilized mechanism[16,23]. As it will be shown in the following sections, there are good relationships between phenolic content values of the extracts and antioxidant activities measured by FRAP and DPPH methods.

Ferric ion reducing capacities of the *F. angulate* extracts have listed in Table 1. The trend for the reducing activities was similar to the TPC measured by FC method. Again, the ML with FRAP value of 360 $\mu mol_{TE}/g_{DM}$ had the highest reducing activity followed by EL, MF and EF. Compared with vegetables such as spinach, broccoli, purple onion, white cabbage, tomato and carrot[24], the ferric reducing potential of the *F. angulata* extracts is markedly greater. However,

TABLE 1: ANTIOXIDANT ACTIVITY OF DIFFERENT EXTRACTS FROM *F. ANGULATA*

Type of extract	TPC value $\mu mol_{TE}/g_{DM}$±SD	FRAP value $\mu mol_{TE}/g_{DM}$±SD	DPPH value $\mu mol_{TE}/g_{DM}$±SD
EL	0.7±0.04	185.4±16.8	321.92±8.8
EF	0.365±0.006	98.7±1.8	264±15.04
ML	0.95±0.03	360±3.1	393.28±1.92
MF	0.565±0.015	142.3±7.8	300.64±4.64

Total phenolic content (TPC value), reducing power (FRAP value) and antiradical activity (DPPH values) of the leaf (EL and ML) and the flower (EF and MF) extracts of *Ferulago angulata*. 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

it must be noted that the solvents used to extract of the antioxidant compounds in the cited study were different from the solvents used in our investigation.

The DPPH assay is technically a simple and reproducible method for evaluating radical scavenging capacities of plant extracts, fruits, olive oil and wine[20]. DPPH radical scavenging activities of the EL, EF, ML and MF extracts were in the range of 260 to 390 $\mu mol_{TE}/g_{DM}$s (Table 1), when they were calculated non-kinetically. Interestingly, similar trends were observed for the FRAP, TPC and DPPH values (ML>EL>MF>EF).

In order to evaluate antiradical behaviours of ML and MF extracts kinetically, the time evolutions of remaining DPPH for each concentration of the extracts were plotted (fig. 1). Depending on the rate of reaching to the steady state, there are three types of kinetic reactions: a rapid kinetics (<1 min), b intermediate kinetics (5-30 min) and c slow kinetics (>1 h)[20]. From the graphs illustrated in fig. 1, it is obvious that antioxidant compounds available in the ML and MF extracts have slow kinetic behaviours. Furthermore, the free radical scavenging activities of ML and MF, representing by EC_{50}, were 0.071 mg/ml and 0.12 mg/ml, respectively. These values are in the range of EC_{50} of known antioxidants such as curcumin, BHT, thymol and carvacrol, having EC_{50} values of 0.0078, 0.02, 0.16 and 0.25 mg/ml, respectively[25].

Among the methods employed in this study, only the ORAC assay is based on hydrogen atom transfer (HAT) mechanism. Besides, in contrast to DPPH assay, the ORAC assay employs a biologically relevant radical[14,23,24], and it is the only method quantifying both inhibition time and degree of inhibition for an antioxidant[21].

Fig. 2 illustrates FL decay curves for Trolox as a standard antioxidant over a concentration range of 0-40 µM. There is a positive correlation between the concentration of Trolox and required time for the decay in fluorescence intensity of FL. 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 EL, EF, ML and MF extracts over concentration ranges of 0-20 µg/ml (fig. 3a-d). From the ORAC values of the extracts (Table 2), calculated by the formula given in the section 2.7, MF showed the highest value (6326 $\mu mol_{TE}/g_{DM}$), while EF had the least (4042 $\mu mol_{TE}/g_{DM}$). Compared to several known extracts, the flower and leaf extracts of *F. angulata* show higher antioxidant

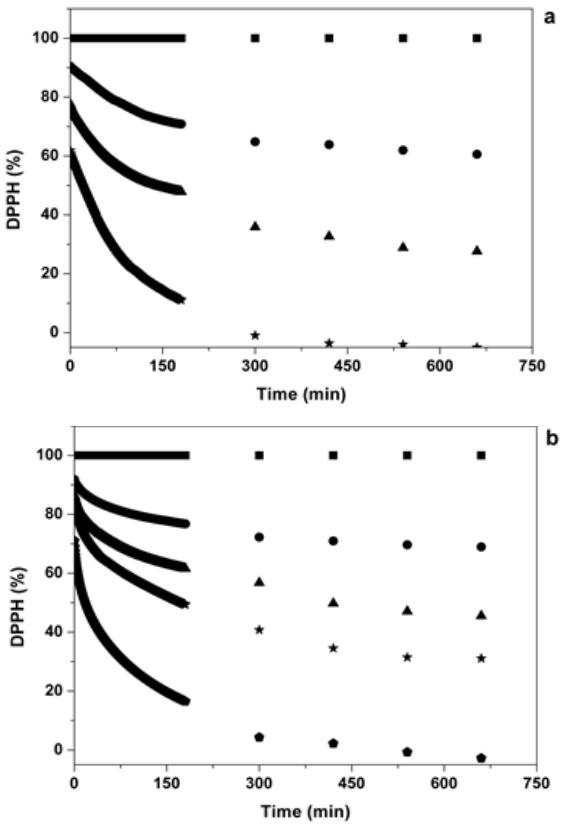

Fig. 1: DPPH kinetic assay
Kinetic curves of DPPH solution in methanol with incremental concentrations of (a) leaf extract (ML) and (b) flower extract (MF) from *F. angulata*. DPPH scavenging capacity of ML is higher than MF in all applied concentrations. ■ Blnk, ● 50 µg/ml, ▲100 µg/ml, * 200 µg/ml, ⌂ 300 µg/ml

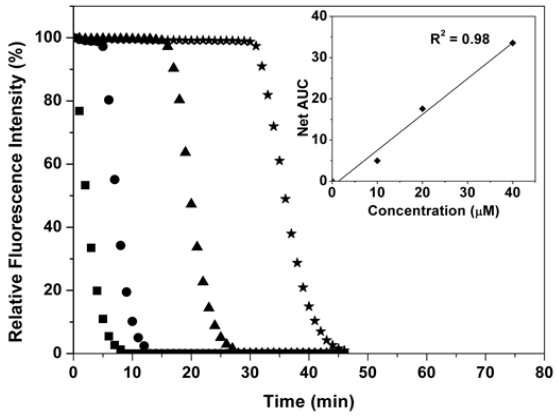

Fig. 2: ORAC assay for Trolox as a standard antioxidant
ORAC assay for Trolox at the indicated initial concentrations, illustrating time-dependent loss of fluorescence of fluorescein's (FL) solutions at presence of AAPH as a biologically relevant radical. With increasing concentrations of Trolox, the time needed for quenching of FL and, consequently, net AUC is increased (inset). ■ Blank, ● 10 µM, ▲ 20 µM, * 40 µM

capacities. For example, extracts from black tea leaves and blueberry have the ORAC values of 1629 and 2792 $\mu mol_{TE}/g_{DM}$, respectively[26], whereas those values for the extracts of *F. angulata* range from 4042

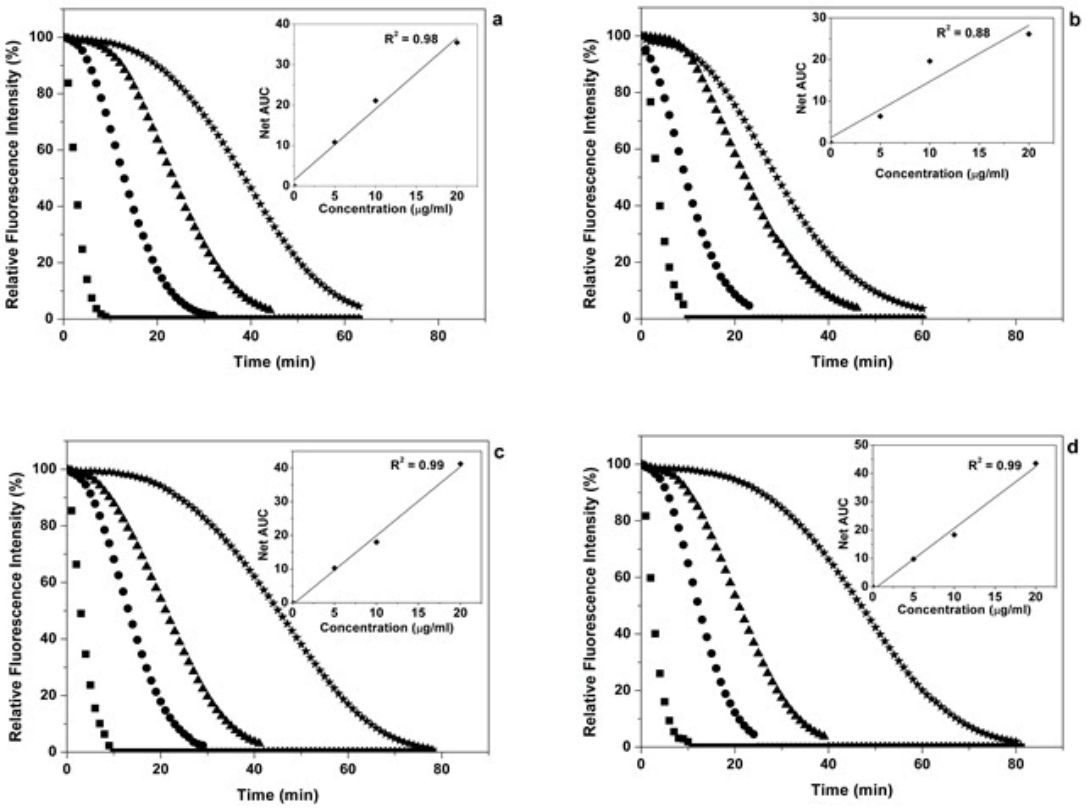

Fig. 3: ORAC-FL curves at presence of *F. angulata* extracts

Fluorescence decay curves of fluorescein at presence of (a) ethanol extract of the leaf (EL), (b) ethanol extract of the flower (EF), (c) methanol extract of the leaf (ML) and (d) methanol extract of the flower (MF) of *F. angulata*. Based on the net AUC profiles (insets on the figure), MF has maximum delaying effect on fluorescein consumption induced by AAPH, and ML, EL and EF occupy next ranks, respectively. Blank, 5 µg/ml, 10 µg/ml, 20 µg/ml

TABLE 2: COMPARISON OF ORAC VALUES BETWEEN DIFFERENT EXTRACTS OF *F. ANGULATA* AND THE WELL-KNOWN EXTRACTS

Type of extract	Extraction solvents (solvent ratio)	ORAC Value $\mu mol_{TE}/g_{DM}$
ML	Methanol (50%):chloroform (1:1)	6176[a]
MF	Methanol (50%):chloroform (1:1)	6326[a]
EL	Ethanol (100%)	5140[a]
EF	Ethanol (100%)	4042[a]
Black tea leaves	Acetone:water (4:1)	1629[26]
Blueberry extracts	Acetone:water (4:1)	2792[26]
Grape skin extracts	Acetone:water (4:1)	15675[26]
Matricaria recutita (flower)	Water	588[24]
Lavandula hybrida grosso (flower)	Water	1181[24]
Actinidia chinensis (flower)	Water	877[24]
Cistus ladaniferus (leaf)	Water	1410[24]

[a]Present study. Comparison of ORAC-FL values in alcoholic extracts of *Ferulago angulata* with some of known extracts in literature. The ORAC values of the flower and leaf extracts of *Ferulago angulata* show higher antioxidant capacities in comparison with the several known extracts. Units of the values are $\mu mol_{TE}/g_{DM}$

to 6326 with the same units (Table 2). However, it was noticed that the methods and the solvents used by Atala *et al.* were different from ours[26]. Notably, *F. angulata* extracts' ORAC values were much lower than that of grape skin with the ORAC value of 15675 $\mu mol_{TE}/g_{DM}$ [21]. This high potential antioxidant activity is also observed for citrus families using different extraction methods[27] and different parts of the orangery[28,29].

Correlations among the methods were calculated by regression analysis on results of various methods used in this study (Table 3). A significant positive correlation was found between FRAP and DPPH assays (R=0.97). Also, results of DPPH and FRAP assays were correlated significantly to the TPC concentration, determined by FC method, with coefficients of 0.97 and 0.88, respectively. These high correlation coefficients indicate that there are good relationships between phenolic compound concentration in *F. angulata* extracts and their antiradical capacity as well as ferric reducing power. In other words, higher phenolic content contributes to a higher antioxidant potential,

TABLE 3: CORRELATION COEFFICIENT

Assay methods	FRAP	DPPH	TPC	ORAC
FRAP	1			
DPPH	0.97	1		
TPC	0.88	0.97	1	
ORAC	0.17	0.4	0.6	1

There are strong correlations between electron-based mechanism methods (FRAP, DPPH and TPC), however, there are weak correlations between ORAC and other methods

a proposition that is reported previously[24,27,28]. Antioxidant potential of phenolic compounds is appertained to number of hydroxyl groups and other substitutions on their aromatic rings[30].

The lowest correlations were found between ORAC and FRAP (R=0.17), ORAC and DPPH (R=0.4) and ORAC and TPC (R=0.6) assays. In contrast to the TPC, DPPH and FRAP assays, the ORAC assay was based on HAT mechanism[23]. Moreover, the ORAC assay is the only method that can be used to evaluate the kinetic behaviors of antioxidants by calculation of area under the decay curves of FL[31]. These differences might explain the low correlation coefficients observed between the ORAC assay and the other methods.

In this investigation, a promising source of natural antioxidant compounds in a rather less studied plant *F. angulate* have been identified. The antioxidant activity of the aerial parts of *F. angulata* was estimated using several well-known methods, such as TPC, FRAP, DPPH and ORAC assays. The first three methods showed high degree of correlation among them for antioxidant activity. These findings suggest that high antiradical potential and reducing power of the alcoholic and hydroalcoholic extracts of the aerial parts of *F. angulata* correspond to a high phenolic content in these plant parts. Additional studies are needed to characterize the active compounds within these plant extracts that may be useful to introduce new nutraceutical agents.

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Conflicts of interest:

There are no conflicts of interest.

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