ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 15:6 (2021) 513-528

records of natural products

Anticholinergic, Antidiabetic and Antioxidant Activities of *Ferula orientalis* L. Determination of Its Polyphenol Contents by LC-HRMS

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(Received February 15, 2021; Revised April 07, 2021; Accepted April 10, 2021)

Abstract: To evaluate the antioxidant activity of evaporated ethanolic extract of *Ferula orientalis* L. (EEFO) and lyophilized water extract of *Ferula orientalis* L. (WEFO) several *in vitro* antioxidant methods such as $ABTS^{\bullet+}$ scavenging activity, $DPPH^{\cdot}$ scavenging activity, Fe^{3+}reduction method, cupric ions (Cu^{2+}) reduction capacity, and metal ion (Fe^{2+})-binding activities using ferrozine reagent were separately performed. Also, BHT, α-tocopherol and ascorbic acid were used as the standard antioxidant molecules. Moreover, some phenolic compounds that are responsible for antioxidant abilities of EEFO and WEFO were determined by LC-HRMS. EEFO and WEFO demonstrated effective antioxidant abilities when compared with the standards. EEFO demonstrated IC_{50} values of 1.946 µg/mL against acetylcholinesterase (AChE), 0.815 µg/mL against α-glycosidase, and 0.675 µg/mL against α-amylase.

Keywords: *Ferula orientalis*; α-glycosidase; antioxidant activity; phenolic compound; acetylcholinesterase, α-amylase.

1. Introduction

Today, herbs are used as a potential resource to develop new products in the cosmetic, food and pharmaceutical industries [1]. *Ferula* is a genus of flowering plants in the Apiaceae family, found in the region from Central Asia to the Mediterranean. It grows mostly in arid climates and having approximately 170 species [2]. *Ferula orientalis* L. is a perennial herb that grows in Central Asia and the Eastern Mediterranean region. It grows on sunny and dry hills, walls, waste soils and limestones and moist soils in the spring. Therefore, this plant can adapt well to the Mediterranean climate and face climatic stresses. It can form large leaf clusters in spring and winter and remaining dormant in mid-

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The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products November-December 2021 EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.236.21.02.1983

summer depending on climatic conditions. In addition, this plant can reach length of about 2 m with numerous yellow flower clusters. It is known that *Ferula orientalis* was used as aphrodisiac, antispasmodic, stimulant, and expectorant effects [2,3].

Antioxidants are natural or synthetic molecules that inhibit oxidation procedure, which produce free radicals and oxidative stress [4,5]. Antioxidants can preserve the human body from the undesired effects of ROS [6,7]. They had beneficial effects in preventing chronic diseases including cardiovascular diseases, Parkinson's disease, cancer, diabetes, Alzheimer's disease (AD), cataracts and the other chronicle disorders. Also, they can terminate the radical chain reactions and neutralize free radicals, which attack cells or biomolecules [8,9]. Vegetables and fruits are healthy foods and rich sources in terms of a large spectrum of natural antioxidants. In this context most antioxidant molecules obtained from natural sources like plants have been found as ROS or free radical scavengers [10,11]. For this reason, alternative, safer, natural and reliable plant-derived antioxidants are preferred as natural antioxidants [12,13].

Antioxidants delay or avoid the onset of major degenerative diseases including diabetes mellitus (DM) and AD [13-15]. One of the main targets in the treatment of DM is the α-glycosidase, the activity of which is fundamental to the degradation of dietary polysaccharides. α-Glycosidase enzyme inhibitors block absorption of monomeric sugar units in the intestinal tract. This limits postprandial plasma glucose level. AGIs can thus be used in the treatment of diabetes and obesity [16,17]. AD is the most typical and common dementia form among elderly people, which negatively affect the personal ability to carry out daily activities. Moreover, it is well known that the loss of cholinergic transmission is one of the major AD causes. Therefore, acetylcholinesterase inhibitors that enhance cholinergic transmission can be used to treat AD. Among them, tacrine is currently used in the palliative treatment for mild to moderate AD. It is known that most of the drugs used today have undesired side effects including hepatotoxicity and gastrointestinal anomalies such as nausea, diarrhea. [18,19]. Especially, it was reported that tacrine had some side effects including vomiting, nausea, stomach upset, weight loss, agitation, diarrhea, shaking, and skin rash [20,21]. Therefore, there is a great demand to develop and use α-glycosidase and AChE inhibitors with antioxidant properties. Phenolic compounds also have anti-AD properties and α-glycosidase inhibition profile. Therefore, one of the most important approaches for treatment of neurodegenerative diseases and DM is natural compounds and products [19, 22-25].

In this study, we aimed to investigation the Cu^{2+} reducing (CUPRAC method), Fe^{3+} reducing and FRAP reducing abilities, $DPPH^{\cdot}$, $ABTS^{\cdot+}$ scavenging effects and Fe^{2+} chelating ability of EEFO and WEFO. An important feature of the study is to quantitatively elucidate some important phenolic contents in the both extracts with LC-HRMS chromatography. Also, another main goal of this study was to determine the possible inhibition effects of EEFO and WEFO against acetylcholinesterase and α-glycosidase enzymes linked to Alzheimer's disease and diabetes.

2. Materials and Methods

2.1. Chemicals

α-Tocopherol, DPPH radical, ABTS and DMPD were purchased commercially from Sigma-Aldrich GmbH (Sternheim, Germany). Ascorbic acid (≥99%, Sigma-Aldrich), (-)-epigallocatechin (>97%, TRC Canada), chlorogenic acid, verbascoside (86.31%, HWI ANALYTIK GMBH), orientin (>97%, TRC Canada), caffeic acid (≥98%, Sigma-Aldrich), luteolin-7-rutinoside (>97%, Carbosynth limited), naringin (≥ 90%, Sigma-Aldrich), luteolin 7-glucoside (>97%, TRC Canada), hesperidin (≥ 98% J&K), rutin (≥94%, Sigma-Aldrich), syringic acid (≥95%, Sigma-Aldrich), rosmarinic acid (≥96 % Sigma-Aldrich), hyperoside (>97% TRC Canada), apigenin 7-glucoside (>97%, EDQM CS), quercitrin (>97%, TRC Canada), quercetin (≥95% Sigma-Aldrich), salicylic acid (≥98%, Sigma-Aldrich), naringenin (≥95%, Sigma-Aldrich), luteolin (95% Sigma-Aldrich), apigenin (>97%, TRC Canada), hispidulin (>97%, TRC Canada), isosakuranetin (>97%, Phytolab), chrysin (≥96%, Sigma-Aldrich), acacetin (>97%, TRC Canada).

2.2. Plant Materials

Ferula orientalis L. samples were gathered from Bingöl province in Turkey, Karlıova district, Kaşıkçı village slopes, at 2030 m altitude (location 39° 24′ 51″ N, 41° 00′ 14″ E, M.Pınar 8971 code) in June 2019. Botanist Assoc. Dr. Süleyman Mesut Pınar identified the plant sample according to the Flora of Turkey [26]. Plant sample was deposited at Van Yuzuncu Yıl University, Faculty of Science, Herbarium of the Biology Department (VANF), Van, Turkey.

2.3. Preparation of Evaporated Ethanolic Extract (EEFO)

Both extraction methods were carried out as described previously [27]. For determination of the ethanolic extract of aerial parts of *Ferula orientalis* L. a 50-g plant sample was cut into small pieces, then, pulverized into a fine powder using a mill and mixed with 500 mL of ethanol and then evaporated [28]. This process was repeated until the extraction solution turned colorless. The combined extracts were filtered (Whatman paper), and evaporated (Heidolph Hei-VAP HL, Germany). Dry ethanolic extract of *Ferula orientalis* L. (EEFO) was transferred to an appropriate plastic bottle and kept at –20 °C until used in experiments.

2.4. Preparation of Lyophilized Ethanolic Extract (WEFO)

For lyophilized water extraction of shade-dried *Ferula orientalis* L.,50-g plant samples powdered and mixed with 500 mL of water, boiled and stirred for 20 min. Then, the extract was filtered and frozen at -87 °C in an ultra-low temperature freezer. Frozen extract was lyophilized at -50 °C at a pressure of 5 mm-Hg in a lyophilizer [29]. Prepared fresh lyophilized ethanolic extract of *Ferula orientalis* L. (WEFO) was kept in a plastic bottle and stored at –20 °C until used in the experiments.

2.5. Reducing Ability

The ferric ion reducing ability of EEFO and WEFO was performed according to Oyaizu [30] as given in previously [31]. For this aim, 2.5 mL of $K_3Fe(CN)_6$ solution (1%), which is prepared in phosphate buffer (0.2 M, 2.5 mL and pH 6.6) was incubated during 30 min. at dark (50 ºC). After that, the same volume of TCA (10%) was transferred to the reaction medium. Then, an aliquot of $FeCl_3$ (0.1%, 0.5 mL) was added and the absorbance was recorded at 700 nm.

The Cu^{2+} ion reducing effects of EEFO and WEFO was made according to spectrophotometric assay of Apak et al. [32] as described previously in detail [33]. EEFO and WEFO were prepared at diverse concentrations (10-30 µg/mL) added to $CuCl_2$ solution (250 µL, 10 mM), Then 250 µL neocuproine solution, which dissolved in ethanol (7.5 mM) and 250 µL of acetate buffer (1.0 M) transferred to the mixture. Finally, after half an hour, the absorbances were recorded at 450 nm.

FRAP reduction ability was determined in accordance with our previous study [34]. Firstly, EEFO and WEFO and standard solutions were transferred to the test tubes, which included several concentrations (50-150 µg/mL). The volumes were supplemented with 0.5 mL of buffer solution, then, a same volume (2250 µL) of $FeCl_3$ (20 mM) and FRAP reactant were transferred to the tubes, respectively. They were vortexed and recorded at 593 nm after a period 10 min.

2.6. Metal Chelating Ability

The metal-binding effect of EEFO and WEFO was performed according to the spectroscopic method of Dinis et al. [35] as described in our previous studies [36,37]. For this process, a solution containing 0.05 mL of $FeCl_2.4H_2O$ (2 mM) and 0.35 mL of deionized water was transferred to 0.2 mL of solution containing EEFO and WEFO. Final volume was compilated to 4 mL with ethanol. Finally, the reaction was started by the addition of 0.2 mL of ferrous solution (5 mM). The solution was vigorously stirred, vortexed and stood at room temperature. After a short incubation period (10 min), the absorbance was spectrophotometrically recorded at 562 nm.

2.7. Radical Scavenging Ability

The DPPH· scavenging ability of EEFO and WEFO was determined in accordance with the method of Blois [38] as given in previous report [39]. Then, stock solutions (1.0 mM) were transferred to the test tubes in differ concentrations (25-75 µg/µL). Finally, total volume was completed to 3 mL with ethanol, then 1 mL DPPH radical solution was added to each test tube and the absorbance was measured at 517 nm.

The $ABTS^{\cdot+}$ scavenging ability of EEFO and WEFO was realized according to the previous study [40]. Primarily an ABTS solution (7.0 mM) was produced by adding to $K_2S_2O_8$ (2.45 nM) and the absorbance was set to 0.700±0.025 at 734 nm upon dilution with buffer solution (0.1 M and pH 7.4). Finally, an aliquot (1 mL) of ABTS radicals was transferred to diverse concentrations (10-30 µg/mL) of EEFO and WEFO (10-30 µg/mL) and the absorbance was recorded at 734 nm.

2.8. Anticholinergic Assay

AChE and BChE inhibitions are used within the scope of anticholinergic studies. The AChE/BChE inhibitory effects of EEFO and WEFO were determined in accordance with Ellman's method [41] as given in previous studies [42,43]. AChE and BChE were commercially purchased and obtained from electric eel (*Electrophorus electricus*) and equine serum, respectively. DTNB and acetylthiocholine iodide/butyrylthiocholine iodide (AChI/BChI) were used as substrates for both cholinergic reactions [44].

2.9. Antidiabetic Assays

The inhibition of two digestive enzymes by EEFO and WEFO was studied within the scope of the antidiabetic study. The efficacy of α-Glycosidase (from *Saccharomyces cerevisiae*) inhibition by EEFO and WEFO was performed according to the method of Tao et al. [45] using *p*-nitrophenyl-D-glucopyranoside (*p*-NPG) substrate and previously described in detail [47]. The absorbances of samples were spectrophotometrically recorded at 405 nm [48]. α-Amylase activity (from porcine pancreas) was determined according to the Xiao's procedure [1]. Starch was used as substrate and prepared in 80 mL NaOH solution (0.4 M, 30 min, and 80 °C).

2.10. Determination of Inhibition Parameters

The IC_{50} was obtained from the activity (%) versus plant concentration plots [49,50].

2.11. Total Phenolic Contents

Total phenolics in EEFO and WEFO were calculated by the Folin-Ciocalteu method [51] as described in a previous study [52]. The quantity of total phenolics in EEFO and WEFO were calculated from the gallic acid calibration curve. Also, total flavonoids in EEFO and WEFO were determined according to a previous colorimetric method [53]. The standard quercetin curve (0-100 µg) was used to determine total flavonoids in EEFO and WEFO. The results are given as µg quercetin equivalents (QE) per g plant extract.

2.12. Preparation of Samples for LC-HRMS Analysis

The dried 50-100 mg of the ethanol-water extracts of plants were dissolved in water in a 5 mL volumetric flask, which was kept in an ultrasonic bath until a clear mixture was occurred. Then, 100 µL of dihydrocapsaicin solution, which used as an internal standard, was transferred and diluted to the volume with mobile phase and stirred and heated to get a clear solution. Then, the solution was filtered (0.45 µm Millipore Millex-HV filter). The concentration of final solution (1 mL) was added in a capped autosampler vial, from which 2 µL of sample was injected to LC for each run. The prepared samples in the auto sampler were stored at 15°C [53-57].

Table 1. LC-HRMS parameters of selected compounds.

Compounds	*m/z*	Ionization mode	Linear range	Linear regression equation	LOD/ LOQ	r^2	Recovery %
Ascorbic acid	175.0248	Negative	0.5-10	y=0.00347x-0.00137	0.39/1.29	0.998	96.2
(-)-Epigallocatechin	307.0812	Positive	0.3-5	y=0.00317x+0.000443	0.17/0.57	0.994	102.2
Chlorogenic acid	353.0878	Negative	0.05-10	y=0.00817x+0.000163	0.02/0.06	0.999	96.7
Verbascoside	623.1981	Negative	0.1-10	y=0.00758x+0.000563	0.03/0.1	0.999	96.2
Orientin	447.0933	Negative	0.1-10	y=0.00757x+0.000347	0.01/0.03	0.999	96.2
Caffeic acid	179.0350	Negative	0.3-10	y=0.0304x+0.00366	0.08/0.27	0.999	94.5
Luteolin-7-rutinoside	593.1512	Negative	0.1-10	y=0.00879x+0.000739	0.01/0.03	0.998	93.1
Naringin	579.1719	Negative	0.05-10	y=0.00576x-0.000284	0.01/0.03	0.999	101.9
Luteolin7-glucoside	447.0933	Negative	0.1-7	y=0.0162x+0.00226	0.01/0.03	0.996	96.3
Hesperidin	609.1825	Negative	0.05-10	y=0.00423x+0.0000138	0.01/0.03	0.999	96.1
Rutin	609.1461	Negative	0.05-10	y=0.00329x-0.00005576	0.01/0.03	0.999	97.0
Syringic acid	197.0456	Negative	0.5-10	y=0.0000831x+0.000024	0.1/0.3	0.999	97.3
Rosmarinic acid	359.0772	Negative	0.05-10	y=0.00717x-0.0003067	0.01/0.03	0.999	99.9
Hyperoside	463.0882	Negative	0.05-10	y=0.0072x-0.00003096	0.01/0.03	0.999	96.6
Apigenin7-glucoside	431.0984	Negative	0.3-7	y=0.0246x+0.00306	0.01/0.03	0.996	96.1
Quercitrin	447.0933	Negative	0.05-10	y=0.0179+0.0003331	0.01/0.03	0.999	97.0
Quercetin	301.0354	Negative	0.1-10	y=0.0509x+0.00467	0.01/0.03	0.997	96.4
Salicylic acid	137.0244	Negative	0.3-10	y=0.0361x+0.00245	0.01/0.03	0.998	92.9
Naringenin	271.0612	Negative	0.1-10	y=0.0281x+0.00182	0.01/0.03	0.999	86.7
Luteolin	285.0405	Negative	0.1-10	y=0.117x+0.00848	0.01/0.03	0.998	96.9
Apigenin	269.0456	Negative	0.3-10	y=0.104x+0.0199	0.01/0.03	0.999	81.5
Hispidulin	301.0707	Positive	0.05-10	y=0.02614x+0.0003114	0.01/0.03	0.999	98.4
Isosakuranetin	285.0769	Negative	0.05-10	y=0.0235x+0.000561	0.01/0.03	0.999	96.6
Chrysin	253.0506	Negative	0.05-7	y=0.0964x-0.0002622	0.01/0.03	0.999	87.9
Acacetin	283.0612	Negative	0.05-7	y=0.046x+0.0001875	0.01/0.03	0.999	87.5

2.13. Instruments and Chromatographic Conditions of LC-HRMS

LC-HRMS experiments were achieved on a Thermo ORBITRAP Q-EXACTIVE mass spectrometery (Bremen Germany) equipped with a Troyasil (Istanbul, Turkey) C18 column (150 x 3 mm i.d., 3 µm particle size). The mobile phases A and B were composed of 1% formic acid-water and 1% formic acid-methanol, respectively. The gradient program of which was 0-1.00 min 50% A and 50% B, 1.01-6.00 min 100% B, and finally 6.01-10 min 50% A and 50% B. The flow rate of the mobile phase was 0.35 mL/min, and the column temperature was set to 22°C. Environmental conditions were set as temperature 22.0 ± 5.0 °C and relative humidity (50 ± 15) % rh [58,59].

2.14. Optimization of HPLC Methods and LC-HRMS Procedure

The best mobile phase was performed to be an acidified methanol and water gradient in HPLC method. This mobile phase was also found to be suitable for ionization abundance and separation of compounds. The best ionization of small and relatively polar compounds was obtained by ESI source. The ions between *m/z* 85-1500 were scanned in high-resolution mode of instrument [11,54-61]. Identification of compounds was performed by comparison of retention time of standard compounds (in the range of purity 95-99% see section chemicals) and HRMS data of Bezmialem Vakif University, Drug Application and Research Center Library (ILMER). Dihydrocapsaicin (purity 95%) was used as an internal standard for LC-HRMS measurements in order to reduce the repeatability problem caused

by external effects, such as ionization repeatability, in mass spectrometry measurements. The detailed mass parameters of each target compound are given in Table 1.

2.15. Method Validation of LC-HRMS Method

Validation of LC-HRMS method was carried out using analytical standards of corresponding compounds (see section 2.1) with using the ions in Table 1 and dihydrocapsaicin was used as an internal standard. Validation parameters are selected as selectivity, linearity, recovery, repeatability, intermediate precision, limit of detection (LOD) and limit of quantification (LOQ). LODs of the method for individual compounds were determined according to the following equation: LOD or LOQ = κSDa/b, where 3 for LOQ and κ= 3 for LOD, SDa represents the standard deviation of the intercept, and b represents the slope. Detailed validation procedure and uncertainty assessment methodology of the applied method was reported in our previous paper [58,59]. Linear range, linear regression equation, r^2, Recovery and LOD and LOQ data are summarized in Table 1.

3. Results and Discussion

Antioxidant and antiradical properties of EEFO and WEFO were carried out in diverse concentrations using different bioanalytical methods such as its (Fe^{2+}) chelating activity, Fe^{3+} reducing ability, Fe^{3+}-TPTZ reduction capacity, Cu^{2+} reduction capability, and ABTS and DPPH radicals scavenging activities. For comparison of antioxidant effects, putative α-tocopherol, BHT and ascorbic acid were used as positive controls. It was also found that the antioxidant activities of both extracts are similar or close to standard antioxidants. Also, it was shown that the antioxidant activity of EEFO and WEFO enhanced with increasing concentrations (10-30 μg/mL). In some cases, the antioxidant ability of EEFO and WEFO was observed to be higher than standard antioxidants at the same concentration. It is well known that the reduction ability is one of the most significant factors in its total antioxidant effectiveness [62]. The antioxidant efficiency can take place through different mechanisms. For example, in a system in which oxidation is accelerated by transition metals, the reduction of antioxidant property of a compound is not significant for antioxidant properties. However, even if the antioxidant only consists of the metal chelating ability, it will stop or slow down the oxidation in such a system. Additionally, electron-withdrawing capacity reflects the reducing ability of a compound [63]. Antioxidants may be in the form of stabilizing oxidants in reductant and redox reactions. The reduction capacity can be recorded by diverse bioanalytical methods. In the presence of reducing compounds, the reduction of ferric complexes ($Fe[(CN)_6]^{3+}$) to the ferrous form ($Fe[(CN)_6]^{2+}$) can readily occur. The addition of Fe^{3+} to the reduced product by addition of EEFO and WEFO leads to the formation of $Fe_4[Fe(CN)_6]$, a complex in the Prussian blue color with sharp absorbance at 700 nm [64].

The increased absorbance shows the increased reduction capacity. As seen in Table 2, EEFO and WEFO exhibited potent Fe^{3+} reducing ability and these diversities were statistically found as considerably important ($p < 0.01$). The reducing capacity of EEFO, WEFO, BHT, α-tocopherol, and ascorbic acid increased constantly when the concentration of sample was increased. Fe^{3+} reducing capacity of EEFO, WEFO and standards exposed the following order: Ascorbic acid (λ_{700}: 1.520±0.028, r^2: 0.9970) > BHT (λ_{700}: 1.269±0.005, r^2: 0.9880) > α-tocopherol (λ_{700}: 0.990±0.007, r^2: 0.9942) > EEFO (λ_{700}: 0.845±0.026, r^2: 0.9390) and WEFO (λ_{700}: 0.645±0.011, r^2: 0.9996) at 20 μg/mL. The results showed that EEFO and WEFO had marked and powerful Fe^{3+} reducing effects.

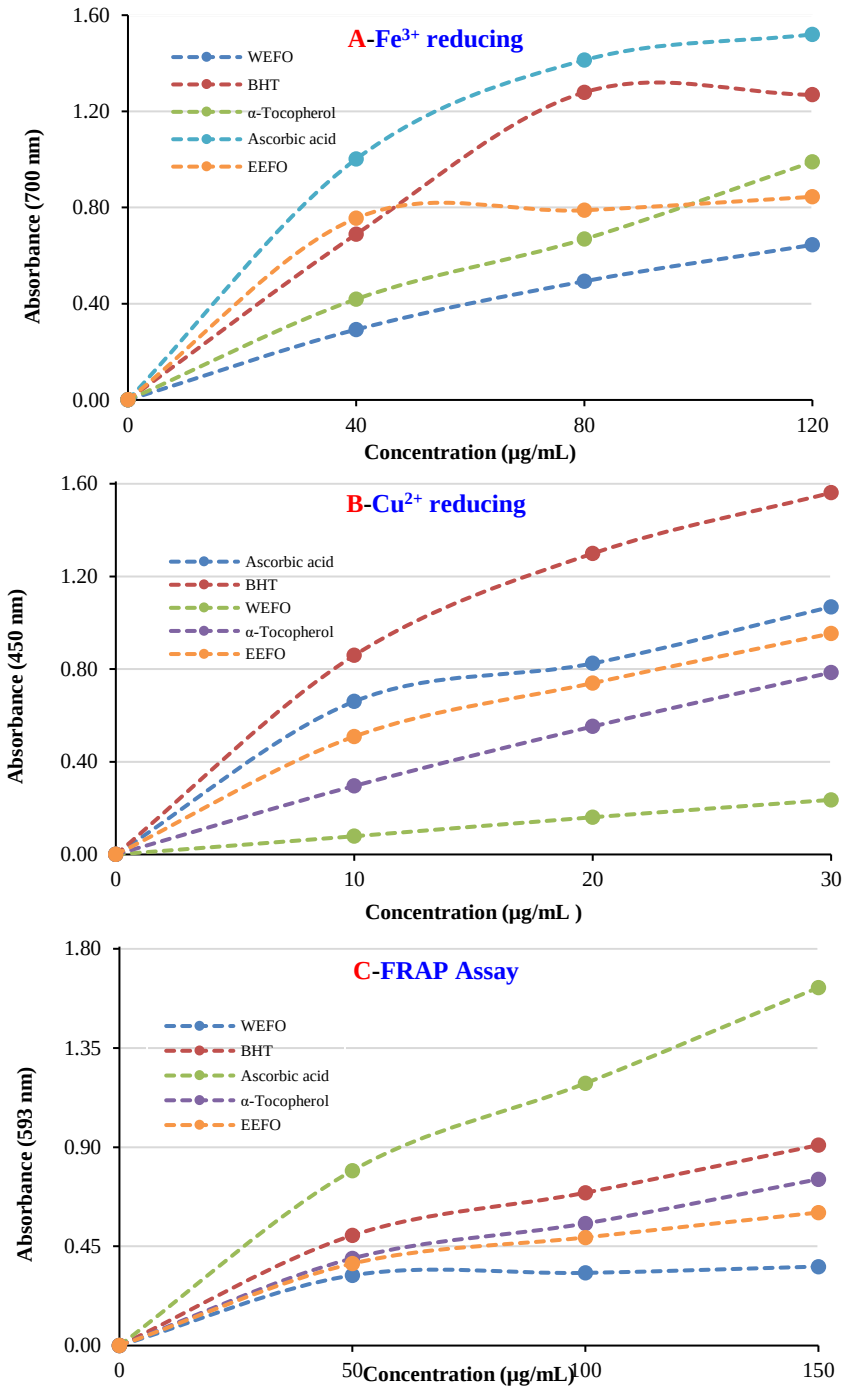


Figure 1. Reducing ability of different concentrations of EEFO, WEFO and standard antioxidants. **A.** Fe^{3+} reducing power. **B.** Cu^{2+} reducing power. **C.** Fe^{3+}-TPTZ reducing power [EEFO: Evaporated ethanolic extract of aerial parts *Ferula orientalis* L. WEFO: Lyophilized water extract of aerial parts of *Ferula orientalis* L., BHT: butylated hydroxytoluene]

Another putative and commonly used method is the reduction ability of Fe^{3+}-TPTZ. It was observed that there was a relationship between the three reduction methods applied to define the reduction capacities of EEFO and WEFO in the studies conducted. Also, it was found that EEFO and WEFO and standard antioxidants showed similar activities in all reduction methods. As can be seen in Table 2, EEFO and WEFO had the effective $Fe^{3+}(CN^{-})_6$, Cu^{2+}-Cu^{+}, and Fe^{3+}-TPTZ reducing abilities, when compared with ascorbic acid, BHT, and α-tocopherol. The results clearly showed that reducing power of EEFO and WEFO and standards decreased in the following order (Table 1): Ascorbic acid (λ_{700}: 1.624±0.015, r^2: 0.9930) > BHT (λ_{700}: 0.909±0.006, r^2: 0.9874) > α-tocopherol (λ_{700}: 0.755±0.075, r^2: 0.9867) > EEFO (λ_{700}: 0.604±0.004, r^2: 0.9843) and WEFO (λ_{700}: 0.358±0.016, r^2: 0.9373). In this assay, ferric ions (Fe^{3+}) are reduced to ferrous ions (Fe^{2+}) in the presence of an antioxidant molecule or extract at the acidic medium (pH 3.6) [65].

Table 2. The reducing power of the EEFO and WEFO and standards antioxidants by Fe^{3+}-Fe^{2+}(120 μg/mL), Cu^{2+}-Cu^{+} (30 μg/mL) and Fe^{3+}-TPTZ (150 μg/mL) reducing methods (EEFO: Evaporated ethanolic extract of aerial parts *Ferula orientalis* L. WEFO: Lyophilized water extract of aerial parts of *Ferula orientalis* L.)

Antioxidants	Fe^{3+}-Fe^{2+} reducing		Cu^{2+}-Cu^{+} reducing		Fe^{3+}-TPTZ reducing	
	λ_{700}	r^2	λ_{450}	r^2	λ_{593}	r^2
α-Tocopherol	0.990±0.007	0.9942	0.785±0.061	0.9986	0.755±0.075	0.9867
Ascorbic acid	1.520±0.028	0.9970	1.069±0.007	0.9722	1.624±0,015	0.9930
BHT	1.269±0.005	0.9880	1.561±0.089	0.9978	0.909±0.006	0.9874
EEFO	0.845±0.026	0.9390	0.954±0.017	0.9928	0.604±0.004	0.9843
WEFO	0.645±0.011	0.9996	0.236± 0.020	0.9998	0.358±0.016	0.9373

Copper is one of putative metals, which can occur in nature in a directly usable metallic form. It is a crucial metal for some endogenous antioxidant enzymes like cytochrome c oxidase [66]. This chromogenic redox reaction measures antioxidants with thiols like non-protein thiols and glutathione. The cupric ions (Cu^{2+}) reducing ability of 20 μg/mL of EEFO, WEFO and standards was shown in Table 2. A positive correlation was observed between the Cu^{2+} reducing ability and different concentrations of the EEFO and WEFO. It was found that Cu^{2+} reducing ability of EEFO and WEFO was occurred in a concentration-dependent manner. Cu^{2+}-reducing ability of EEFO and WEFO and standards at the same concentration (30 μg/mL) demonstrated the following order: BHT (λ_{700}: 1.561±0.089, r^2: 0.9978) >ascorbic acid (λ_{700}: 1.069±0.007, r^2: 0.9722) > α-tocopherol (λ_{700}: 0.785±0.061, r^2: 0.9986) > EEFO (λ_{700}: 0.954±0.017, r^2: 0.9928) and WEFO (λ_{700}: 0.236± 0.020, r^2: 0.9998).

Table 3. The half maximum concentration (IC_{50}, μg/mL) of EEFO, WEFO and standards for the DPPH, DMPD, ABTS radicals scavenging activities (EEFO: Evaporated ethanolic extract of aerial parts *Ferula orientalis* L. WEFO: Lyophilized water extract of aerial parts of *Ferula orientalis* L.)

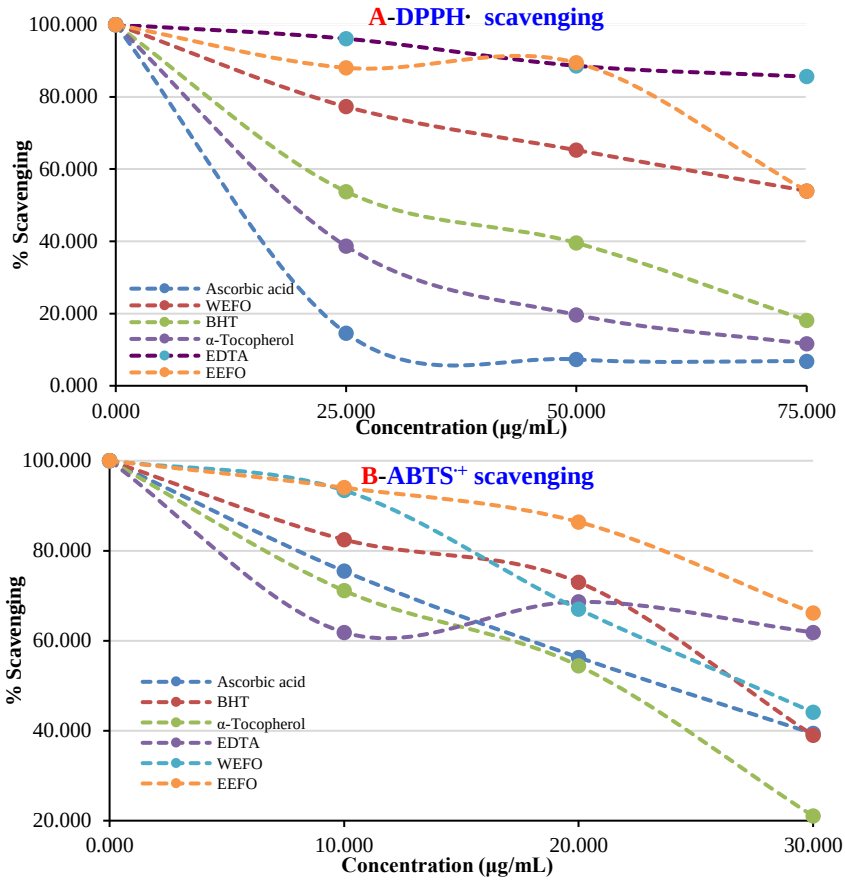
Compounds	DPPH• scavenging		$ABTS^{\bullet+}$scavenging		Metal chelating	
	IC_{50}*	r^2	IC_{50}*	r^2	IC_{50}*	r^2
α-Tocopherol	30.1	0.9578	8.1	0.9606	33.0	0.9006
Ascorbic acid	17.3	0.9509	7.5	0.9519	99.0	0.9985
BHT	38.5	0.9858	5.8	0.9539	14.7	0.9647
EEFO	346.5	0.8507	90.9	0.9901	4.39	0.9334
WEFO	86.6	0.9916	17.3	0.9968	-*	-*

*They were not determined.

The iron-chelating ability is very significant due to ionic species like ferrous ion (Fe^{2+}) facilitating the production of free ROS in the organism [67]. This binding assay is a significant antioxidant method used for prevention or delaying of oxidation processes catalyzed by metal ions, however excessive metal ions can cause cell damage. Among the metal ions, ferrous ions (Fe^{2+}) are the most significant prooxidant ions. It is a significant lipid oxidizing metal due to it has high activity in transition metals. Fe^{2+} ions are more reactive than Fe^{3+} ions [68]. These reactions can also occur OH

radicals, which are more reactive than the end-peroxides. Metal-binding effects of EEFO and WEFO was evaluated using two distinct metal chelator agents including ferrozine reagent [69]. When the IC_{50} values of the binding effect of EEFO and WEFO in the study were compared with the IC_{50} of the EEFO, WEFO and standard antioxidants was found as effective metal chelator with IC_{50}: 4.39 µg/mL (r^2: 0.9334, Table 3) using ferrozine reagent, however, this value could not be determined for WEFO. Also, relatively higher IC_{50} values were found for α-tocopherol (IC_{50}: 33.0 µg/mL, r^2: 0.9006), ascorbic acid (IC_{50}: 99.0 µg/mL, r^2: 0.9985), and BHT (IC_{50}: 14.7 µg/mL, r^2: 0.9647).

The radical scavenging is very significant in terms of damage to the organism by free radicals and ROS in living organisms. Recently, many distinct methods have been developed for the removal of ROS and free radicals. Thus, they reduce the quality of the food and pharmaceutical products [70]. The spectrophotometric methods based on the radical scavenging are frequently used for determination of antioxidant abilities of substances, beverages, food, and herbal extracts. In addition, $ABTS^{\cdot+}$ and DPPH· scavenging methods are fast, simple, selective and repeatable procedures. So, they are widely used to define the radical elimination abilities. It is easy to use the violet DPPH· and green-blue $ABTS^{\cdot+}$ chromogens, which have high sensitivity [71]. As seen in Table 3, within the scope of DPPH free radical scavenging studies, IC_{50} values for EEFO and WEFO had less effective DPPH· scavenging and were found to be 346.5 µg/mL (r^2: 0.8507) and 86.6 µg/mL for ascorbic acid (r^2: 0.9916). As with DPPH radical scavenging ability, $ABTS^{\cdot+}$ scavenging ability is extensively used for determination of radical scavenging activities of beverages, extracts and substances [69]. $ABTS^{\cdot+}$ is more reactive radical than DPPH radical. As shown in Table 3, it is observed that EEFO and WEFO had effective ABTS radical removing effects. The IC_{50} value of $ABTS^{\cdot+}$ scavenging activity for EEFO and WEFO was calculated as 90.9 µg/mL (r^2: 0.9901) and 17.3 µg/mL (r^2: 0.9968), respectively. Also, this value was counted 5.8 µg/mL for BHT (r^2: 0.9539), 8.1 µg/mL for α-tocopherol (r^2: 0.9606) and 7.5 µg/mL for ascorbic acid (r^2: 0.9606). The results clearly demonstrated the EEFO and WEFO have effective, but lower $ABTS^{\cdot+}$ scavenging ability than that of all standard antioxidants.



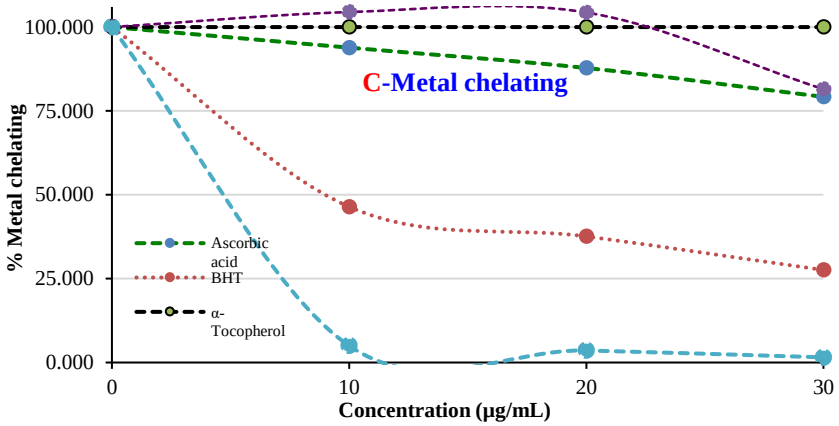


Figure 2. A) DPPH radical scavenging activity. **B)** ABTS radical scavenging activity. **C)**Metal chelating activity [EEFO: Evaporated ethanolic extract of aerial parts *Ferula orientalis* L. WEFO: Lyophilized water extract of aerial parts of *Ferula orientalis* L., BHT: butylated hydroxytoluene]

Acetylcholinesterase (AChE) is another important metabolic enzyme and had been associated in some neurodegenerative diseases such as AD and Parkinson diseases [72]. The AChE inhibition had a positive effect on the long-term progression of AD. In this context, there are many published studies on the inhibition effects of compounds and crude extracts. One such compound is galantamine and used to treat mild AD to moderate AD and firstly isolated from *Galanthus woronowii* [73]. Also, MEFO effectively inhibited AChE with IC_{50} values of 1.946 µg/mL (r^2: 0.9752) and for AChE. On the other hand, tacrine was used as positive control for AChE inhibition, and had K_i value of 0.124 µM (r^2: 0.9804) against AChE, which is the primary cholinesterase at mainly neuromuscular junctions and in chemical synapses in the body [74]. However, it was observed that WEFO had not any effects against the used metabolic enzymes.

Similarly, EEFO had effective inhibition against α-glycosidase with IC_{50} values of 0.815 µg/mL (r^2: 0.9525). Tacrine was used as positive control for both cholinergic enzyme inhibition, and had K_i value of 0.101 µM (r^2: 0.9698) against AChE. Aside from these inhibition properties, EEFO had demonstrated marked inhibition effect against α-amylase, which is an extracellular enzyme and hydrolyzes glycogen and starch to glucose units, with IC_{50} values of 0.675 µg/mL (r^2: 0.9706).

Until recently, diabetes has remained one of the fastest growing, serious and costly health problems in the world. Its complete cure and effective drugs for treatment are still not available [75]. Recently, herbal extracts and their compounds have received great attention as antioxidants and potential inhibitors of key and metabolic enzymes, used in clinical conditions. For example, α-glycosidase and α-amylase as essential digestive enzymes in carbohydrate metabolism has been considered a key target for reducing postprandial hyperglycemia in diabetic patients.

In this context, it has been reported that biologically active compounds like acarbose, voglibose and miglitol reduce postprandial hyperglycemia by inhibiting enzymes that carry out carbohydrate digestion, thereby delaying or partially inhibiting glucose absorption [76]. A recent research has been conducted directly towards the discovery of amylase inhibitors of naturally effective ingredients and extracts with potential use as therapeutic agents for the treatment of diabetes and prevention of diseases. Human saliva α-amylase is the most plentiful digestive enzyme in human saliva that hydrolyzes polysaccharides such as starch to oligosaccharides [75]. EEFO had IC_{50} value of 0.68 µg/mL (r^2: 0.9706) toward α-glycosidase and 0.82 µg/mL against α-amylase enzyme (r^2: 0.9525) (Table 3). The results show that EEFO as a crude extract exhibited efficient α-glycosidase and α-amylase inhibition effect than that of acarbose, which had IC_{50} of 10.00 µM for α-amylase, 22.80 µM for a-glucosidase. The results clearly showed that EEFO had more effective inhibition than that of acarbose, which was a starch blocker [77].

Total phenolic compound in EEFO and WEFO was determined using the Folin–Ciocalteu reagent. Gallic acid, which is easily obtained in large amounts by acid or alkaline hydrolysis of tannin, was used for a standard graph (r^2: 0.9840). Plants, vegetables and fruits are important phenolic

compounds sources of human diet. Accordingly, the consumption of foods including polyphenols had a great importance for their natural antioxidants [68]. The quantity of phenolics in EEFO and WEFO was determined using the equation taken from standard gallic acid graph as gallic acid equivalents (GAE/mg extract). On the other hand, for determination of total flavonoids content of EEFO and WEFO, a standard quercetin chart was used. The quantity of flavonoids was determined with the equation of standard flavonoid graph. It was shown that 4.9 and 6.7 µg of QE flavonoids were found in 1 mg of EEFO and WEFO, respectively.

Table 4. The quantity (mg/kg extract) of phenolic antioxidant compounds in EEFO and WEFO determined by LC-HRMS chromatograms.

Compounds	WEFO	EEFO	U (%)
Ascorbic acid	139.41	<LOD	3.94
(-)-Epigallocatechin	<LOD	0.04	3.09
Chlorogenic acid	7409.23	0.73	3.58
Verbascoside	4.62	<LOD	2.93
Orientin	<LOD	<LOD	3.67
Caffeic acid	149.17	0.02	3.74
Luteolin-7-rutinoside	<LOD	<LOD	3.06
Naringin	3.61	<LOD	4.20
Luteolin 7-glucoside	<LOD	<LOD	4.14
Rutin	15.74	<LOD	3.07
Rosmarinic acid	71.30	0.09	3.77
Hyperoside	1864.20	0.18	3.46
Apigenin 7-glucoside	<LOD	<LOD	3.59
Quercitrin	33.73	<LOD	3.78
Quercetin	4.79	0.01	2.95
Salicylic acid	4.79	<LOD	1.89
Naringenin	<LOD	<LOD	4.20
Luteolin	0.65	<LOD	3.42
Apigenin	<LOD	0.01	2.87
Hispidulin	<LOD	<LOD	3.41
Isosakuranetin	<LOD	<LOD	3.98
Chrysin	5.09	<LOD	3.24
Acacetin	3.25	0.03	3.98
Fumaric acid	<LOD	0.16	2.88

EEFO: evaporated ethanolic extract of roots and aerial parts *Ferula orientalis* L.
WEFO: lyophilized water extract of roots and aerial parts of *Ferula orientalis* L.)

Also, the standard chromatogram for phenolic compounds by LC-HRMS (mg/mL) is given in Figure 1. According to LC-HRMS analysis, the main phenolic compounds identified in WEFO are chlorogenic acid (7409.23 mg/kg), hyperoside (1864.20 mg/ kg) and caffeic acid (149.17 mg/kg). On the other hand, chlorogenic acid (0.73 mg/kg), hyperoside (0.18 mg/kg) and (-)-epigallocatechin (0.04 mg/kg) are the most abundant phenolics in 1 mg of EEFP (Table 4). Phenolic compounds, in other words polyphenols, are predominantly of plant origin. They give color to the plants. The plants protect themselves using polyphenols against many external influences. The antioxidant property of polyphenols is well established and well known [78]. Phenolic compounds have biological functions such as free radical scavenger and metal chelator. In plants, the antioxidant effects of phenolics are mainly due to redox effects. For this reason, hydrogen donors, reducing agents, singlet oxygen inhibitors and metal chelates act as builders [54].

4. Conclusion

The determination of the bioactivity and screening of phytochemical of EEFO and WEFO had great importance. Both extracts were evaluated for their bioactivities contain antioxidant activities and some

metabolic enzymes inhibitory properties associated with several global diseases. Generally, the high action of the EEFO was in proportional to its phenolic content when compared to WEFO. Indeed, many studies have also found that a positive correlation between total phenolic and flavonoid contents of plant extracts and their biological efficiencies. In addition, when the LC-HRMS results are evaluated, it is clearly seen that the main phenolic compounds responsible for the antioxidant and other biological activities of both extracts are chlorogenic acid, hyperoside, and caffeic acid. Ethanol was observed as effective solvent for the extraction of phenolics with effective antioxidant activity and inhibition of α-glycosidase, AChE and α-amylase enzymes. Nowadays, enzyme inhibition to control overactive enzyme activities has become a key target in the treatment or management of many chronic diseases, especially AD, cancer and diabetes.

Acknowledgments

S. Alwasel would like to extend his sincere appreciation to the Researchers Supporting Project (RSP-2021/59), King Saud University, Saudi Arabia.

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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