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Acetylcholinesterase inhibitory activities and LC-MS analysis of the antioxidant *Ferula caspica* M. Bieb. and *F. halophila* Peşmen extracts

Çiğdem KAHRAMAN [1]*, İpek BAYSAL [2], İ. İrem ÇANKAYA [3], Fatih GÖGER [4,5,6], Neşe KIRIMER [6], Zeliha Ş. AKDEMİR [1]

[1] Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey.
[2] Department of Biochemistry, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey.
[3] Department of Pharmaceutical Botany, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey.
[4] Department of Pharmacy, Program in Pharmacy Services, Yunus Emre Vocational School, 26470 Eskişehir, Turkey.
[5] Anadolu University Medicinal plants, Drugs and Scientific Research Centre (AUBIBAM), 26470 Eskişehir, Turkey.
[6] Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, 26470 Eskişehir, Turkey.
* Corresponding Author. E-mail: cigdemm@hacettepe.edu.tr (Ç.K.); Tel. +90-544-622 77 27.

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ABSTRACT: The aim of the current study is to evaluate the biological activity of *F. caspica* M. Bieb. which is used for the treatment of diabetes in traditional Turkish medicine and to compare with *F. halophila* Peşmen. Antioxidant capacities and total phenolic contents of the plants' extracts were tested by DPPH radical scavenging assay and Folin Ciocalteu assay, respectively. Acetylcholinesterase inhibitory activities of the extracts were investigated with the aim of indicating whether there was a correlation with their antioxidant capacities. Different quinic acid and flavonoid derivatives which may be responsible for the activities of the extracts were detected using LC-MS screening in the methanol extracts. The methanol extract from the aerial parts of *F. caspica* was found to show most antioxidant capacity and acetylcholinesterase inhibitory activity.

KEYWORDS: *Ferula*; antioxidant capacity; acetylcholinesterase inhibition; LC-MS.

1. INTRODUCTION

The genus *Ferula* L. is the third largest genus of Apiaceae [1] represented by 23 species, 13 of which are endemic to Turkish Flora [1-8]. *Ferula* species are wealthy natural biological sources for developing new drug design due to traditional usage for treatment of several disorders in Anatolia as well as around the world. Some species are used as aphrodisiac, antidiabetic and anticholesterolemic agent in Turkey [9]. Besides that, antispasmodic [10, 11], antidiabetic and antihyperlipidemic [12, 13], cytotoxic [14, 15] and antimicrobial [16] activities of different *Ferula* species were reported previously. The aerial parts of *F. caspica* M. Bieb. is also traditionally used as antidiabetic in the East Anatolian region of Turkey [9].

Free radicals are products with high reactivity containing unpaired electrons such as hydroxyl radical ($^{\bullet}OH$), superoxide anion ($O_2^{\bullet -}$) and nitric oxide ($NO^{\bullet}$) or non-radical reactive molecules derived from free radicals such as peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2). Though these reactive oxygen species are produced by natural biochemical processes or external effects, oxidative stress damaging the biological molecules such as lipids, proteins, polysaccharides, and DNA occurs when the balance between free radicals production and antioxidant defense mechanisms deteriorates [17-19]. Such imbalance has an effect on the pathogenesis of Alzheimer's disease (AD), Parkinson's disease (PD) and other neurodegenerative diseases [20, 21]. Among them, AD identified by the abnormal deposition of amyloid β (Aβ) peptide, and intracellular accumulation of neurofibrillary tangles is a common, progressive and neurodegenerative disease characterized by loss of memory and other cognitive functions [22, 23]. Plasma antioxidant levels were found to be significantly lower in AD patients [24, 25].

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The aim of the present work to determine the antioxidant capacities and phenolic compound profiles by LC-MS as well as the relationship between acetylcholinesterase (AChE) inhibitory and antioxidant activities of the extracts from *F. caspica* and *F. halophila*.

2. RESULTS

2.1. DPPH radical scavenging capacity assay

DPPH radical scavenging capacity assay is one the most practical, rapid and common method used for determined the antioxidant activity. In this electron transfer based assay [26], the reduction of 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) by an hydrogen-donating antioxidant is identified by measuring the decrease of absorbance ($DPPH^{\bullet} + A\text{-}H \rightarrow DPPH\text{-}H + A^{\bullet}$). Composing reduced DPPH-H by transferring the proton to $DPPH^{\bullet}$ from antioxidant (A-H) results in the decolorization of the radical [27].

The methanol extracts of *F. caspica* and *F. halophila* showed higher antioxidant capacities compared to other extracts in DPPH radical scavenging assay (Table 1).

Table 1. DPPH radical scavenging capacity of *F. caspica* and *F. halophila* extracts.

Extracts	% Inhibition			
	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
FC/AP/$CHCl_3$	11.62 ± 0.56	14.27 ± 0.64	17.35 ± 0.73	28.16 ± 1.32
FC/AP/MeOH	10.04 ± 0.95	16.26 ± 0.52	27.16 ± 0.67	52.21 ± 0.55
FC/Root/PE	3.53 ± 0.32	6.62 ± 0.20	8.02 ± 0.36	13.26 ± 0.68
FC/Root/$CHCl_3$	6.19 ± 0.71	12.33 ± 0.87	19.14 ± 0.11	31.19 ± 0.68
FC/Root/MeOH	4.70 ± 0.56	9.90 ± 0.75	14.93 ± 1.22	28.04 ± 0.74
FH/AP/PE	-	-	4.41 ±0.22	6.56 ±0.26
FH/AP/$CHCl_3$	-	6.88 ± 1.74	9.11 ± 2.96	12.16 ± 2.43
FH/AP/MeOH	7.30 ± 0.08	16.21 ± 0.42	28.48 ± 0.72	53.17 ± 6.71
FH/Root/PE	-	-	-	-
FH/Root/$CHCl_3$	3.87 ± 2.43	6.36 ± 1.52	6.17 ± 1.39	7.32 ± 1.12
FH/Root/MeOH	-	4.46 ± 0.43	13.78 ± 1.52	21.21 ± 0.52
Quercetin	70.21 ± 1.42	90.65 ± 0.08	90.84 ± 0.88	92.01 ± 0.33

FC: *F. caspica*, FH: *F. halophila*, AP: Aerial parts, $CHCl_3$: Chloroform extract, PE: Petroleum ether extract, MeOH: Methanol extract.

2.2. Determination of total phenolic content

The basis of the method is that the spectrophotometric measurement of blue color produced by reduction of the molybdenum center in the Folin-Ciocalteu reagent while the electron donating phenolic compound is oxidized [28]: $Mo(VI) + e \rightarrow Mo(V)$

Since the FC reagent is nonspecific to the phenolic compounds, the basic conditions with sodium carbonate are provided to allow the phenolic compounds to react [26]. The results showed that the methanol extract contains the higher phenolics with the value of 59.16 ± 3.42 mg gallic acid equivalent/g extract in comparison with chloroform extract (46.51 ± 2.03 mg GA equivalent/g extract) (Table 2).

2.3. Aluminium chloride colorimetric assay (Total Flavonoid Assay)

The spectrophotometric measurement of the intensity of the chelates composed between $AlCl_3$ and C-4 keto group and the C-3 or C-5 hydroxyl groups or ortho hydroxyl groups in the A- or B-ring of the flavonoids is the principle of the assay. The results are given in Table 2.

Table 2. Total phenolic and flavonoid contents of *F. caspica* and *F. halophila* extracts.

Extracts	Total Phenol mg GA/ g extract	Total Flavonoid mg Quercetin/ g extract
FC/AP/$CHCl_3$	46.51 ± 2.03	8.17 ± 0.34
FC/AP/MeOH	59.16 ± 3.42	12.14 ± 1.67
FC/Root/PE	42.62 ± 1.85	-
FC/Root/$CHCl_3$	90.33 ± 2.94	-
FC/Root/MeOH	36.94 ± 2.36	-
FH/AP/PE	21.38 ± 2.03	6.98 ± 1.20
FH/AP/$CHCl_3$	44.84 ± 2.53	33.88 ± 3.44
FH/AP/MeOH	67.37 ± 3.16	30.79 ± 1.13
FH/Root/PE	8.05 ± 1.07	-
FH/Root/$CHCl_3$	14.53 ± 1.18	-
FH/Root/MeOH	18.11 ± 1.11	-

2.4. LC–MS analysis of phenolic compounds

Especially, sesquiterpene and coumarin derivatives were isolated from apolar extracts of *Ferula* species [29-32]. Studies on the polar metabolites are unfortunately limited. The present research focused on the extracts showing antioxidant properties and is important for the preliminary screening of polar metabolites (Table 3) which can be responsible for the activity. 5-Caffeoylquinic acid was found to be the major compound in *F. caspica* and *F. halophila* aerial part extracts as shown in Figures 1-3.

Table 3. Monitored compounds in methanol extracts by LC-MS.

Rt min	$[M-H]^-$ *m/z*	Fragment	Compounds	Extracts	Ref
8.9	353	191, 179, 135	3-Caffeoylquinic acid	FC/AP, FH/AP- Root	[46]
10.2	353	191, 179,173, 135	5-Caffeoylquinic acid	FC/AP-Root, FH/AP-Root	[46]
11.2	515	335, 191, 179, 161, 135	1,3-Dicaffeoylquinic acid	FH/Root	[46]
12.7	367	191, 173	5-Feruloylquinic acid	FC/AP-Root,	[46]
13.6	463	301, 271, 255, 179, 151	Quercetin-3-*O*-*β*-glucoside	FC/AP, FH/AP	[47]
14.5	515	353, 335, 191, 179, 173, 161, 135	3,4/4-5-Dicaffeoylquinic acid	FC/AP	[46]
14.7	153	109	Dihydroxy benzoic acid	FC/AP-Root,	[43]
14.8	447	285, 255, 227	Kaempferol-3-*O*-*β*-glucoside	FC/AP, FH/AP	[48]
15.2	353	191, 179, 173, 135	Caffeoylquinic acid deriv.	FH/AP	[46]
15.4	515	353, 191, 179	1,5-Dicaffeoylquinic acid	FC/Root, FH/Root	[46]
15.9	515	353, 191, 179, 173	3,4/4-5-Dicaffeoylquinic acid	FC/AP-Root FH/AP	[46]
17.1	431	285, 255 ,227	Kaempferol-3-*O*-*a*-rhamnozide	FC/AP, FH/AP	[45]
18.2	529	367	Caffeoylferuloylquinic acid deriv.	FH/AP	[46]
19.5	193	179, 161, 133	Dihydroxycinnamic methyl ester deriv.	FH/AP	[44]
21.2	301	179, 151	Quercetin/Hesperidin	FH/AP	[49]

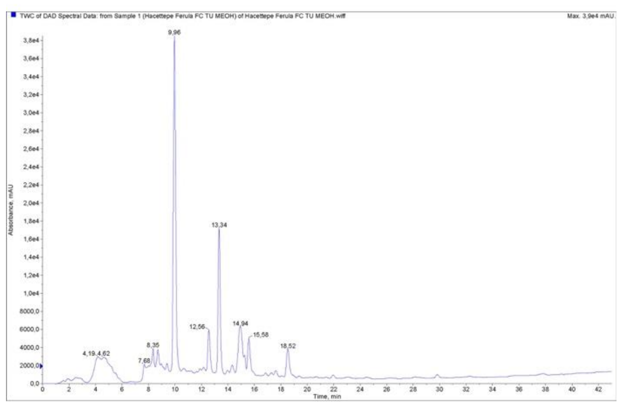

Figure 1. HPLC-DAD chromatogram of methanolic extract from the aerial parts of *F. caspica*

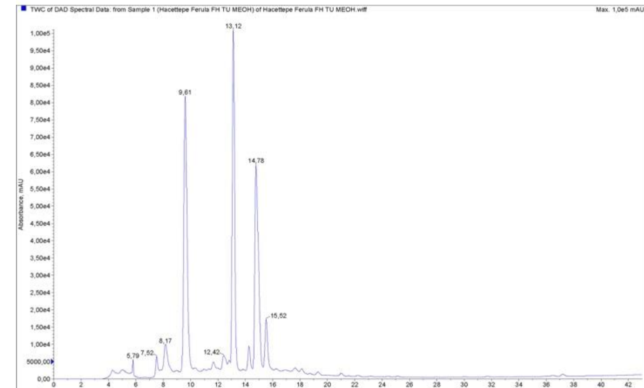

Figure 2. HPLC-DAD chromatogram of methanolic extract from the aerial parts of *F. halophila*

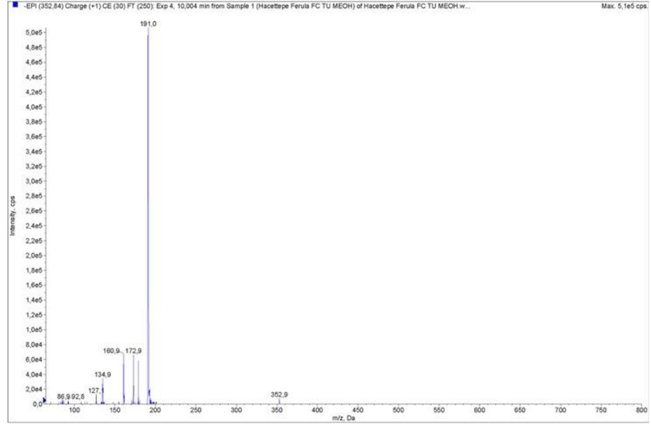

Figure 3. Mass spectrum of 5-caffeoylquinic acid.

2.5. Acetylcholinesterase inhibitory activity

Acetylthiocholine forms thiocholine (as well as acetate) in the presence of acetylcholinesterase enzyme, gives yellow color with dithiobisnitrobenzoic acid. Enzyme inhibition is based on the reduction of yellow color and the measurement of color intensity spectrophotometrically [33]. As enzyme inhibition results in an increasing cholinergic function, AChE inhibitors featured in the symptomatic treatment of Alzheimer Disease. Methanolic extract from the aerial parts of *F. caspica* demonstrated higher activity in comparison with donepezil as the standard (Table 4).

Table 4. Acetylcholinesterase inhibitory activities of the *F. caspica* and *F. halophila* extracts.

Extracts	IC_{50} (µg/ml)	Extracts	IC_{50} (µg/ml)
FC/AP/$CHCl_3$	84.37636 ± 6.1306	FH/AP/$CHCl_3$	0.87385 ± 0.0672
FC/AP/MeOH	**0.04433** ± 0.0268	FH/AP/MeOH	**0.28759** ± 0.1564
FC/Root/PE	0.36386 ± 0.2021	FH/Root /PE	8.59918 ± 0.9382
FC/Root/$CHCl_3$	106.04545 ± 12.8565	FH/Root /$CHCl_3$	25.33879 ± 5.9761
FC/Root /MeOH	4.45797 ± 1.2348	FH/Root /MeOH	148.70455 ± 17.9027
FH/AP/PE	19.21394 ± 0.0847	Donepezil	0.00008 ± 0.0000065

3. DISCUSSION

The methanol extracts of both species showed antioxidant activity in DPPH radical scavenging assay. Both extracts mostly contained different quinic acid and flavonoid derivatives which may be responsible for the activities. These phenolic compounds have antioxidant activities because of their reductant potentials of their phenolic hydroxyl groups. Since one-electron reduction potentials and reactivities of the phenolic

radicals are lower than those of the reactive oxygen species (ROS), free radicals can be easily reduced by phenolic hydroxyl. Thus, phenolic compounds have radical scavenge activity on destructive radical species [34]. Position of the hydroxyl groups is a determinative factor of antioxidant capacities of the molecules as well as the second hydroxyl group in the ortho- position increases the antioxidant activity [35].

Oxidative damage is associated with aging and is widespread in the brain in Alzheimer disease. So, the extracts were studied for a correlation between antioxidant capacity and acetylcholinesterase inhibitory activities. The antioxidant methanol extract of the aerial parts of *F. caspica* showed the highest AChE inhibitory activity compared to other extracts.

In recent studies, *F. halophila* and *F. elaeochytris* methanol extracts displayed potent antioxidant capacities in comparison with apolar extracts [36, 37]. Besides, in acetylcholinesterase inhibitory activity studies, the chloroform extracts of *F. persica* var. *persica* and *F. halophila*, as well as the hexane extract of *F. elaeochytris*, were assigned to be more active than methanolic extracts of the plants [36-38]. In addition, the acetylcholinesterase inhibitory activities of coumarin derivatives from *F. gummosa and F. asafoetida* species were determined, previously [30-41]. In the current study, the chloroform extracts of *F. caspica* and *F. halophila* that could be expected to contain some coumarin derivatives weren't found to be as active as methanol extracts of the plants compared to standard donepezil. The low activity of the chloroform extracts can be explained by the differences in the extraction procedures and the intensity of the active ingredients in the extracts.

Several studies in diabetes, demonstrated free radicals are increased by some reactions derived from the development of the disease and defense against radicals decreases [12, 13]. In addition, the activity of antioxidant enzymes in pancreatic homogenates from diabetic animals has been shown to decrease with oxidative stress [17, 42], however, further studies are needed to prove this claim for *F. caspica* and *F. halophila* extracts.

3. CONCLUSION

This is the first report to compare the phytochemical properties and activities of the roots and aerial parts of these two species. The methanol extracts from the aerial parts of *F. caspica* and *F. halophila* were found to show antioxidant and acetylcholinesterase inhibition activities. The activities complied with the total phenolic and flavonoid contents of the extracts and these results were associated with the content of secondary metabolites such as quinic acid and flavonoid derivatives of both species studied. In the present study, the higher AChE inhibitory activities of the methanol extracts gained from *F. caspica* and *F. halophila* can be explained by their higher antioxidant activities and phenolic compounds. However, high total phenolic content of the chloroform extract from the roots of *F. caspica* wasn't correlated with its antioxidant and acetylcholinesterase inhibitory activities.

4. MATERIALS AND METHODS

4.1. Plant materials

F. caspica was collected from Nallıhan-Davutoğlan Bird Paradise in June 2011 and *F. halophila* was collected from Şereflikoçhisar-Kaldırım Salina in June 2013, Ankara province Central Anatolia Region of Turkey. Voucher specimens were identified by Prof. Dr. Hayri Duman (Gazi University, Faculty of Science, Ankara, Turkey) and were deposited at the Herbarium of the Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 11003 and HUEF 13002).

4.2. Extraction

The aerial parts of *F. caspica* (10 gr) were extracted with chloroform (100 ml x 3) and methanol (100 ml x 3), respectively, using a rotary extractor under 40 °C without vacuum. After the roots of *F. caspica* and *F. halophila*, as well as the aerial parts of *F. halophila* (10 g), were extracted with petroleum ether (100 ml x 3), chloroform (100 ml x 3) and methanol (100 ml x 3), respectively. All of the obtained extracts were tested for antioxidant capacity, total phenolic content, acetylcholinesterase inhibitory activity and used for the analysis of the phenolic profile by liquid chromatography-mass spectrometry (LC–MS).

4.3. Antioxidant capacity determined using the DPPH assay

The free radical scavenging activity of the methanolic extract was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) according to the previously reported by Brand-Williams et al. [27] with slight modifications. After 50 μL of 1mM DPPH solution was added to the 150 μL of methanol extract prepared at

the concentration of 200-25 µg/ml and shaken gently with a vortex mixer, the mixtures were incubated in dark at room temperature for 30 min. The decrease in absorbance of mixtures was measured at 517 nm against blank using a UV spectrophotometer. Inhibition % values of the extracts and reference compound were calculated using the formula below:

$$\text{Inhibition \%} = [(A_{blank} - A_{extract/reference}) / A_{blank}] \times 100 \quad \text{(Eq. 1)}$$

A_{blank} expresses the absorbance of the mixture of all reagents without antioxidants. Quercetin was used as the reference compound and the calculated inhibition % values of the extracts are given in Table 2.

4.4. Determination of total phenolic content

A slight modification of Folin-Ciocalteu assay described by Slinkard and Singleton [43] was used for the determination of phenolic content. According to method, 100 µL of water diluted Folin-Ciocalteu reagent (1:10) was added to 20 µL of methanol extract prepared at the concentration of 1000-500 µg/ml. After the mixture was shaken gently, 80 µL of Na_2CO_3 (7.5 %) was added and the mixture was stored at room temperature for 2 h. The absorbance was measured at 765 nm against blank and total phenolic content was determined using the standard calibration curve of gallic acid and expressed as gallic acid equivalents (Table 2).

4.5. Determination of total flavonoid content

For determination of total flavonoid content of the extracts, Aluminium Chloride Colorimetric Assay [44] was used 0.5 ml solutions in different concentrations of the test materials were diluted with 1.5 ml of 95% ethanol, 0.1 ml of 10% $AlCl_3$, 0.1 ml of 1 M Potassium Acetate (CH_3COOK) and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance was measured at 415 nm against the blank. The quantity of flavonoids was determined using the standard calibration curve of quercetin and results were expressed as mg quercetin equivalent per g extract (Table 2).

4.6. LC–MS analysis of phenolic compounds

Shimadzu 20A HPLC system, Absciex 3200 Q-Trap MS-MS detector and 250 x 4.6 mm and analytic colon containing 5 µm octadecyl silica were used for LC-MS/MS identification of phenolic compounds.

5 µL of methanol extract was injected to an octadecyl silica column and eluted with the mixture of mobile phase A:Acetonitrile:water:formic acid (10:89:1) and mobile phase B:Acetonitrile:water:formic acid (89:10:1) at a flow rate 0.5 ml min^{-1}. Column oven was set at 40 ºC and the gradient began at 10% (B) and increased to 90% (B) during 40 min. LC-ESI-MS / MS data were collected and processed with Analyst 1.6 software. The results are compared with the literature [45-51] and the possible definitions are given in Table 3.

4.7. Acetylcholinesterase inhibitory activity

Acetylcholinesterase inhibitor activity was measured by using Ellman's method [33]. In 96-well plates, phosphate buffer (500 mM, pH 7.4), acetylthiocholine iodide (5mM), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (0.125 mM) and 7 mg/ml-16 µg/ml concentrations of the extracts were pre-mixed and the enzymatic reaction was started with the addition of the acetylcholinesterase. Absorbance changes were measured at 412 nm by using a microplate reader. To evaluate the IC_{50}, log concentration versus percentage of inhibition curve was plotted and analyzed using GraphPad Prism (Table 4).

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