REPORT

Phytochemical analysis, antimicrobial, antioxidant activities and total phenols of *Ferulago carduchorum* in two vegetative stages (flower and fruit)

Fereshteh Golfakhrabadi[1], Mohammad Reza Shams Ardekani[1,2], Soodabeh Saeidnia[3], Fatemeh Yousefbeyk[4], Hossein Jamalifar[5], Nasrin Ramezani[1], Tahmineh Akbarzadeh[6,2] and Mahnaz Khanavi[1,7]*

[1]Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Traditional Pharmacy, Faculty of Traditional Medicine and Persian Medicine and Pharmacy Research Center, Tehran University of Medical Sciences, Tehran, Iran
[3]Medicinal Plants Research Center, Tehran University of Medical Sciences, Tehran, Iran
[4]Department of Pharmacognosy, School of Pharmacy, Guilan University of Medical Sciences, Rasht, Iran
[5]Department of Drug and Food Control, Faculty of Pharmacy and Pharmaceutical Quality Assurance Research Center, Tehran University of Medical Sciences, Tehran, Iran
[6]Department of Medicinal Chemistry, Faculty of Pharmacy and Drug Design & Development Research Center, Tehran University of Medical Sciences, Tehran, Iran
[7]Faculty of Land and Food Systems, University of British Columbia, Vancouver, BC, Canada

Abstract: *Ferulago carduchorum* (Apiaceae family) is an endemic plant of Iran. The crude extract and four fractions of aerial parts of *F. carduchorum* in two vegetative stages (flower and fruit) were studied for their total phenolic contents, antimicrobial and antioxidant activities using folin-ciocalteu assay, micro dilution method and DPPH assay, respectively. The results indicated that the best antioxidant activity was determined in flower crude extract (IC_{50}=0.44mg/mL). The flower ethyl acetate fraction (FLE) showed better antimicrobial and antifungal activities than other fractions. So, FLE was selected for phytochemical investigations, resulting in isolation of a flavonoid (hesperetin). Hesperetin showed antimicrobial activity. The results showed that the antimicrobial and antioxidant effects during the flowering are obviously more than the fruit season.

Keywords: *Ferulago carduchorum*, DPPH assay, phenolic content, antimicrobial activity.

INTRODUCTION

In recent years, the worldwide trend of using natural antioxidants is increasing (Frankel, 1993; Velasco and Williams, 2011) and natural antibiotics are still used for treat infectious diseases (Uysal *et al*., 2005). Synthetic antioxidants exhibit side effects, so many efforts have been made to achieve a variety of natural antioxidants (Khanahmadi and Janfeshan, 2006). Some studies suggest that natural antioxidants are able to reduce the risk of cardiovascular diseases and several cancers (Xia *et al.*, 2011). The majority of studies over the antioxidant and antimicrobial activities in plants have focused on phenolic compounds (Jafari*et al*., 2010) and many coumarin derivatives (Patel Rajesh and Patel Natvar, 2011; Basile *et al.,* 2009). *Ferulago carduchorum* Boiss. & Hausskn. (Apiaceae) is an endemic plant of Iran, which disperses in west part of Iran (Mozaffarian, 2007), where it has been traditionally added to dairy and oil ghee to delay expiration date and give them a pleasant taste. Additionally, in the past, this plant was used as natural preservative to increase the shelf life of meat. In Turkey a number of *Ferulago* species have been used as sedative, tonic, aphrodisiac, remedy of digestive pains and hemorrhoid (Sodeifian and Bamoniri, 2011). Furthermore some species of this genus are benefit for diseases of spleen, headache, ulcers and snake-bites (Demetzos *et al.*, 2000). In previous phytochemical studies on *Ferulago* species, different coumarins were recognized. In previous studies on *F. carduchorum* (Golfakhrabadi *et al*., 2014a), *F. bernardii* (Khalighi-Sigaroodi *et al.*, 2006), *F. turcomanica* (Andrianova *et al.*, 1975; Serkerov *et al.*, 1976), *F. asparagifolia* (Doganca *et al*., 1992), *F. nodosa* (Ruberto *et al.*, 1994), *F. capillaris* and *F. Brachyloba* (Jimenez *et al.,* 2000)the presence of coumarins were revealed. There are also some reports about the acetyl cholinesterase inhibitory (Dall'Acqua *et al*., 2010), cytotoxic (Rosselli *et al*., 2009), antimicrobial and antioxidant activities of coumarins isolated from *F. campestris* (Basile *et al*., 2009). Moreover, chemical

**Corresponding author:* e-mail: khanavim@sina.tums.ac.ir

composition and biological effects of *F. Carduchorum* essential oil have been reported (Golfakhrabadi *et al*., 2014b).

In this study the antimicrobial and antioxidant activities as well as total phenolic contents of the extracts and fractions of *F. carduchorum* in two vegetative stages (flower and fruit) were investigated base on folk uses. Also phytochemical analysis was carried out on the most potent fraction.

MATERIALS AND METHODS

Instruments

The ^{1}H and ^{13}C-NMR spectra were recorded on a Brucker Avance TM 500 DRX spectrometer that it was 500 MHz for ^{1}H and 125 MHz for ^{13}C.Tetramethylsilanewas used as an internal standard and chemical shifts were given in δ (ppm) and the coupling constants (J) are in Hz. The UV spectra were obtained using a Shimadzu 160A spectrophotometer. The silica gel 60F254 precoated plates (Merck TM) were used for thin layer chromatography (TLC). The spots were detected under UV (254 and 365 nm) and by spraying anisaldehyde-H_2SO_4 reagent followed by heating. Silica gel for column chromatography (Mesh 70-230) was purchased from Merck Company (Germany).

Plant material and extraction

The aerial parts of *F. carduchorum* in flower and fruit vegetative stages were collected from mountains of Illam, province of west of Iran at June and August 2011, respectively. The voucher specimen is deposited in herbarium of Institute of Medicinal Plants (ACECR). The samples were dried separately at room temperature (17-22°C) and were crushed into appropriate size. The air-dried and ground aerial parts of *F. carduchorum* in two vegetative stages (flower and fruit) were extracted separately by percolation method with MeOH/H_2O (80/20) three times at room temperature. The fractions were provided respectively with hexane, ethyl acetate, methanol, methanol/H_2O (50/50). The extracts and fractions were evaporated by rotary evaporator and freeze-dried. Then, they were stored in refrigerator to investigate the antioxidant and antimicrobial activities and total phenolic content.

The flower ethyl acetate fraction (20g) was selected for phytochemical studies. This fraction was subjected to silica gel column chromatography (CC) with hexane: AcOEt (9:1, 0:1) and MeOH as eluents to give eight fractions (E1- E8). The fraction E5 (4 g) was submitted to silica gel CC with hexane, AcOEt (7:3, 0:1) to obtain nine fractions (E51-E59). Pure compound was obtained from fraction E59 (316mg) by silica gel CC and hexane-AcOEt (7:3, 0:1) as mobile phase.

DPPH radical scavenging activity

The DPPH radical scavenging activity was measured using by the method described by Yokozawa *et al*. (Yokozawa *et al*., 1998). 1mL of different concentrations of extracts and fractions (or MeOH as control) were mixed 2mL DPPH (Merck, Germany) solution and the absorbances were measured at λmax 517 nm every 5min up to 30 min. The experiment was repeated three times. Percentage of radical scavenging activity of extracts was calculated by using the equation: inhibition% = [(A0-As)/A0]*100 that A0 is the absorbance of the control and As is the absorbance of the sample. The antioxidant activity was reported as IC_{50} (the required concentration of the compound (mg/mL) that can be scavenge 50% of DPPH), obtained from a linear regression analysis (Yousefbeyk *et al*., 2014a).

Measurement of the total phenolic contents

Total phenolics were determined colorimetrically using Folin-Ciocalteu reagent as described by Khanavi *et al*. (Khanavi *et al*., 2009). The total phenolic contents were calculated by the calibration curve obtained from measuring the absorbance of Gallic acid (GA) concentration as standard (20-150µg/mL). The results were expressed as equivalent milligrams of Gallic acid (GA) per 1 g dry extract.

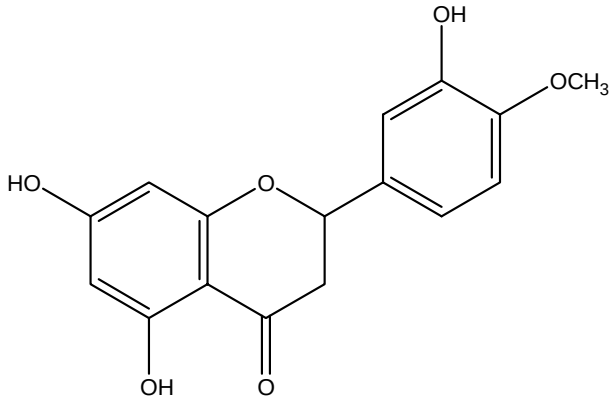

Fig. 1: Molecular structure of isolated flavonoid (hesperetin) from flower ethyl acetate fraction (FLE) of *Ferulago carduchorum*

Antimicrobial activity

Antimicrobial activities of the crude extracts and fractions of *F. carduchorum* in two vegetative stages (flower and fruit) were determined against both Gram-positive (*Staphylococcus aureus* ATCC 6538), Gram-negative (*Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027) bacteria and a fungal strain (*Candida albicans* ATCC 1023) by micro dilution method. The dilutions of extracts and fractions ranged from 125 to 0.243mg/ml concentrations in microtiter plates. Dimethyl sulphoxide (DMSO) was used as negative control, which was solvent used to dissolve the extracts. Nystatin was used as positive control against *C. albicans* while, Ampicillin was used as positive control against *S. aureus*,

Table 1: Antioxidant activity and total phenol contents (as mg GAE/ 1g dry extract) of *Ferulago carduchorum* extract and fractions in two vegetative stages

Samples	Extraction %	Radical DPPH inhibition %	IC_{50} mg/mL	Total phenol contents (as mg GAE/ 1g dry extract)
FLC	22.08	86.77±3.46	0.49	60.29±0.033
FLH	1.01	-	9.4	-
FLE	2.29	45.82±5.51	1	-
FLM	12.39	66.96±7.48	0.68	69.31±0.048
FLM50%	17.88	73.56±1.59	0.78	51.97±0.043
FRC	25.83	67.95±3.37	0.62	54.89±0.01
FRH	0.8	-	28.21	-
FRE	1.5	42.81±6.09	1.25	-
FRM	9.44	55.57±1.3	0.92	52.51±0.014
FRM50%	10	50.4±2.59	0.94	57.55±0.032
Vitamin E	-	-	0.015	-

Note: The reported percentages for inhibition% were related to concentration of 1mg/mL extracts and fractions. flower crude extract (FLC), flower hexane fraction (FLH), flower ethyl acetate fraction (FLE), flower methanol fraction (FLM), flower methanol 50% fraction (FLM50%), fruit crude extract (FRC), fruit hexane fraction (FRH), fruit ethyl acetate fraction (FRE), fruit methanol fraction (FRM) and fruit methanol 50% fraction (FRM50%).

E. coli and *P. aeruginosa.* Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the fractions were assessed by broth micro dilution method with visible growth observed by using 96 U-shaped wells plates (NCCLS, 2006). The endpoint of MIC is the lowest concentration of the sample at which the test strain does not show visible growth. The MBC was determined by quantitative subculture of 100µl from each clear well onto MHA and SDA agar plates. Plates were incubated at 37°C (bacteria) and 20-25°C (*C. albicans*) for 48 h. The MBC is defined as the lowest of extract that results in more than 99.9% killing of the bacteria being tested (Vazirian *et al.*, 2012; Yousefbeyk *et al*., 2014b).

STATISTICAL ANALYSIS

All experiments (total phenolic contents, antimicrobial and antioxidant activities) were done three times and the data were reported as mean ± SD. One-way ANOVA and Tukey post-hoc multi comparison tests were exerted for the analyses and comparing of samples radical scavenging activity with vitamin E. Also, these analyses used for comparing antimicrobial activity of extracts with positive controls (Ampicillin and Nystatin). Statistically significance level was $p<0.05$.

RESULTS

In this study, antimicrobial, antioxidant activity and total phenol contents of *F. carduchorum* in 2 vegetative stages (flower and fruit) were determined. Yields of the crude extracts and fractions are shown in table 1. The following two crude extracts and eight fractions including flower crude extract (FLC), flower hexane fraction (FLH), flower ethyl acetate fraction (FLE), flower methanol fraction (FLM), flower methanol 50% fraction (FLM50%), fruit crude extract (FRC), fruit hexane fraction (FRH), fruit ethyl acetate fraction (FRE), fruit methanol fraction (FRM) and fruit methanol 50% fraction (FRM50%), have been employed in this study. The results of total phenol and DPPH assay are shown in table1. Vitamin E also used as a reference compound to compare IC_{50} of all the samples.

As shown in table 2, all the fractions had antimicrobial activity against *Staphylococcus aureus*. Ampicillin and nystatin also used as positive controls to compare MICs of all the samples. DMSO used as a negative control. Evaluation of MIC and MBC of all extracts showed that the FLE demonstrated the best antimicrobial effect against both Gram-positive (*S. aureus*), Gram-negative (*E. coli* and *P. aeruginosa*) bacteria and a fungal strain (*C. albicans*) (MIC=1.95, 15.62, 31.25, 3.90mg/mL, respectively) in comparison with the other fractions. So, FLE was selected for isolation and characterization of active compounds by silica gel column chromatography. Isolated compound was identified as flavonoid hesperetin (fig. 1) by resemblance of its NMR spectral data with previous reports in literature (Maltese *et al.*, 2009). Pure compound (Hesperetin) is yellow powder.

^{1}H-NMR (DMSO-d6): 6.94 (bs, 1H, H-5'), 6.93 (d, 1H, J=2 Hz, H-2'), 6.87 (dd, 1H, J=8.3 Hz, 2, H-6'), 5.90 (d, 1H, J=2 Hz, H-8), 5.89 (d, 1H, J=2 Hz, H-6), 5.43 (dd, 1H, J=12.3, 3 Hz, H-2), 3.77 (s, 3H, OCH_3), 3.20 (dd, 1H, J=17.2, 12.3 Hz, H-3_{eq}), 2.71 (dd, J=17.2, 3 Hz, 1H, H-3_{ax}), ^{13}C-NMR (DMSO-d6): 196.23 (C-4), 166.68 (C-7), 163.50 (C-5), 162.83 (C-9), 147.91 (C-4'), 146.48 (C-3'), 131.17 (C-1'), 117.71 (C-6'), 114.08 (C-2'), 111.98 (C-5'), 101.83 (C-10), 95.02 (C-8), 95,83 (C-6), 78.25 (C-2), 42.09 (C-3) (Maltese *et al*. 2009).

Table 2: Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) of extract and fractions of flower and fruit against selected bacteria and *C. albicans*

	Staphylococcus aureus		*Escherichia coli*		*Pseudomonas aeruginosa*		*Candida albicans*	
Fractions	MIC[b]	MBC[b]	MIC	MBC	MIC	MBC	MIC	MBC
FLC	3.90	7.81	-[a]	-	-	-	-	-
FLE	1.95	3.90	15.62	31.25	31.25	62.5	3.90	7.81
FLM	7.81	15.62	-	-	31.25	62.5	-	-
FLM50%	7.81	15.62	-	-	-	-	-	-
FLH	1.95	3.90	15.62	31.25	-	-	7.81	15.62
FRC	7.81	15.62	-	-	-	-	-	-
FRE	1.95	3.90	15.62	31.25	31.25	62.5	7.81	15.62
FRM	7.81	15.62	-	-	62.5	125	-	-
FRM50%	15.62	31.25	-	-	-	-	-	-
FRH	3.90	7.81	31.25	62.5	-	-	7.81	15.62
Hesperetin	0.468	0.937	-	-	7.5	15	0.937	1.875
Ampicillin Nystatin DMSO (Negative control)	0.25×10^{-3} - -	0.35×10^{-3} - -	0.064 - -	0.124 - -	0.016 - -	0.032 - -	- 0.008 -	- 0.016 -

Note: [a]Not effective. [b]MIC and MBC were determined by broth micro dilution method and expressed in mg/mL. flower crude extract (FLC), flower hexane fraction (FLH), flower ethyl acetate fraction (FLE), flower methanol fraction (FLM), flower methanol 50% fraction (FLM50%), fruit crude extract (FRC), fruit hexane fraction (FRH), fruit ethyl acetate fraction (FRE), fruit methanol fraction (FRM) and fruit methanol 50% fraction (FRM50%).

Hesperetin showed antimicrobial activity against *S. aureus*, *P. aeruginosa*, *C. albicans* with MIC= 0.47, 7.5, 0.94mg/mL, respectively (table 2).

DISCUSSION

This evaluation is the first study on antibacterial, total phenol and antioxidant activity of *F. carduchorum* extracts and fractions in two stages (flower and fruit), however biological effects of *F. carduchorum* essential oil have been reported, recently (Golfakhrabadi *et al*., 2014b).

These results of antimicrobial activity indicated that pure compound had the lower MICs than crude extracts and fractions but it showed antimicrobial effects less than positive controls (Ampicillin and Nystatin), ($P<0.05$).According to results of antimicrobial activity the MICs of extracts and all fractions were more than positive controls ($P<0.05$). FLE demonstrated the best antimicrobial effect in comparison with the other fractions. The antimicrobial effect of FLE may be due to content of flavonoids like hesperetin, which was isolated from this fraction. The FLH indicated noticeable efficiency against *S. aureus* (MIC=1.95mg/mL). All the fractions showed moderate activity against Gram-positive bacteria but had less active against the Gram-negative bacteria. Antimicrobial effect during the flowering is more than the fruit season. Antimicrobial studies on 3 species of *Ferulago* from Greece, showed that the hexane extracts had MICs >800µg/mL against Gram-positive and negative bacteria (Demetzos *et al*., 2000) that MICs were less than FLH and FRH. In a previous study, antimicrobial activity of *F. carduchorum* essential oil was investigated against *S. aureus*, *E. coli*, *P. aeruginosa* and *C. albicans* and MICs values were less than 23mg/mL (Golfakhrabadi *et al*., 2014b). So, the essential oil of *F. carduchorum* had more potent antimicrobial activity than its extract and fractions. In this research radical scavenging activity of FRC, FLM, FRM 50% and FLM50% at 1mg/mL and FLC at 0.5mg/mL were comparable with vitamin E (0.02mg/mL), ($P>0.05$). The results showed that the inhibitory effects of crude extract in fruit and flower vegetative stages were more potent than their fractions (IC_{50}=0.62 and 0.49mg/mL, respectively). It was demonstrated that extract and fractions of flower phase had the higher radical scavenging capacity than fruit ones. Among tested samples, the highest capacity of inhibition was observed in FLC (IC_{50}=0.49mg/mL). The highest amount of phenol content was measured in FLM as 69.31±0.04mg gallic acid equivalent (GAE) / 1g dry extract. In flower and fruit vegetative phases methanol and methanol–aqueous fractions (69.31±0.04 and 57.55±0.03mg GAE /1g extraction, respectively) had highest phenol content. The total phenol content in flower extract and fractions was more than fruit vegetative phase. Correlations between antioxidant activity and phenolic content in extracts and different fractions in flower and fruit stage were $y = 1.2033x-29.864$ ($R^2=0.3548$).

In according to past study on antioxidant activity of extract of *F. angulata* in vegetable oil, *F. angulata* had good antioxidant potential (Khanahmadi and Janfeshan,

2006). Another study of Apiaceae family showed *F. carduchorum* possesses good capacity of inhibition in DPPH assay. But the amount of phenolic compounds is less than other plants. For example in *Ferula assafoetida* IC_{50} for DPPH radical-scavenging activity was 380mg/mL. The total phenolic content of this species (94.8±5.9mg GAE/g of extract powder) was higher than *F. carduchorum* (Dehpour *et al.*, 2009). In one study on *Prangos* species revealed that their DPPH radical-scavenging activity was less than extracts and fractions of *F. carduchorum.* Also, majority of *Prangos* species had higher amount of total phenolic content in comparison with *F. carduchorum* (Ahmed *et al.*, 2011).

These results state that the phenolic content cannot be only responsible for the antioxidant activity of *F. carduchorum.* A number of species that can be mentioned as natural antioxidants in Apiaceae family are as follows: *Pimpinella anisum*, *Trigonella foenum*, *Coriandrum sativum*, *Anethum graveolens* and *Foeniculum vulgare* Miller (Souri *et al*., 2003). Otherwise based on results and folk uses, *F. carduchorum* could be natural antioxidant.

CONCLUSION

In Conclusions, *F. carduchorum* collected from west of Iran can be consider as a potential source a source of natural radical scavenger and antimicrobial to be used in the food and pharmaceutical industries. The results showed that the antimicrobial and antioxidant effects during the flowering stage are obviously more than the fruit season. The MIC value of pure compounds may be noticeably lower in comparison with crude extract, so isolation and characterization of these agents is needed to determine antibacterial effect of them. These process as well as synthesis of new agents for investigating antibacterial activity is beneficial to find new antibiotics.

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