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An investigation on antioxidant and antimicrobial activities of four Inula helenium L. taxa

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Abstract

The antioxidant activities of methanol (ME), ethanol (EE), water (WE) and ethyl acetate (EAE) extracts of four *Inula helenium* L. taxa (*I. helenium* ssp. *orygalis* (Boiss.) Grierson, *I. helenium* ssp. *pseudohelenium* Grierson, *I. helenium* ssp. *turcarasemosa* Grierson and *I. helenium* ssp. *vanensis* Grierson) were investigated. The phosphomolybdenum assay, β-carotene–linoleate bleaching and DPPH radical scavenging activity were used to evaluate the antioxidant capacity. The total phenolic contents determined by Folin-Ciocalteu assay of the extracts ranged from 4.18 to 102.91 mg gallic acid equivalents (GAE)/g dry extract. The extracts showed considerable effect on reducing the oxidation of β-carotene. The highest radical scavenging activity was obtained for ME of *I. helenium* ssp. *orygalis* in DPPH assay. ME, EE and EAE of four *Inula helenium* taxa showed significant antibacterial activity against 13 bacteria tested. WEs had no inhibitory effect against bacteria tested except for *I. helenium* ssp. *orygalis*. EE of *I. helenium* ssp. *orygalis* was only effective against *C. albicans*.

Keywords*: Inula helenium*; antimicrobial activity; antioxidant activity; DPPH

1. Introduction

Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants (Dai and Mumper, 2010). Plant polyphenols comprise a diversity of compounds, among which flavonoids and several classes of nonflavonoids are usually distinguished. More than 4000 flavonoids have been identified in plants, and the list is constantly growing (Cheynier, 2005). Flavonoids have many diverse functions including defense, UV protection, auxin transport inhibition, allelopathy, and flower coloring (Buer, 2010). They have been reported to possess many useful properties, including anti-inflammatory, oestrogenic, enzyme inhibition, antiallergic, antioxidant, vascular and cytotoxic antitumour activities (Buer, 2010; Cushnie and Lamb, 2005). The antimicrobial effects of polyphenols have also been widely reported and there is an increasing interest in this topic because plant polyphenols could represent a source of new anti-infective agents against antibiotic-resistant human pathogens (Ferrazzano et al., 2011).

Oxygen metabolism continuously generates small amounts of reactive oxygen species (ROS). ROS are normally produced during physiologic processes such as cellular respiration and inflammatory defense mechanisms (Crimi et al., 2006). Generation of ROS beyond the antioxidant capacity of a biological system gives rise to oxidative stress. Oxidative stress plays a role in the pathogenesis of several diseases (Krishnaiah et al., 2007). Increasingly, plant polyphenols are becoming the subject of medical research due to their marked effects in the prevention of various oxidative stress associated diseases such as cancer (Dai and Mumper, 2010).

The genus *Inula*, a variable perennial herb distributed in Asia, Europe and Africa, comprises ca. 100 species of the Compositae (Asteraceae) family. *Inula* species are rich sources of sesquiterpenes and monoterpenes (Bai et al., 2005; Zhao et al., 2010). Several species in this genus are used as traditional herbal medicines throughout the world for treatment of many diseases, including bronchitis, diabetes, intestinal ulcers, digestive disorders and inflammation (Bai et al., 2005; Zhao et al., 2010). They were reported to have anti-inflammatory, antibacterial, antihepatitic and antitumor activities (Bai et al., 2005). *I. helenium* is used topically in folk medicine as anti-scabies and to promote wound-healing. Thus, it is named as "Scabwort" (Akay, 2002). In the literature, there are a limited number of studies on the *Inula* species growing in Turkish flora (Karamenderes and

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Zeybek, 2000; Akay, 2002; Gençaslan, 2007; Sengul et al., 2009).

The purpose of this study was to investigate the total phenolic contents, total antioxidant capacities, ability to inhibit the bleaching of the β-carotene and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities and, antimicrobial activities of methanol, ethanol, water and ethyl acetate extracts of four *I. helenium* taxa growing in Turkey.

2. Materials and methods

2.1. Chemicals

Folin-Ciocalteu reagent, DPPH, sodium carbonate, gallic acid, ascorbic acid, nutrient agar, nutrient broth, malt extract agar and malt extract broth were purchased from Merck (Darmstadt, Germany). The other chemicals and solvents used in this experiment were analytical grade, also purchased from Merck.

2.2. Plants

The collection information of the *I. helenium* taxa, which are individually numbered, is listed below:

1. *I. helenium* ssp. *orgyalis*, between Eflani and Daday, Kastamonu, 1137 m, 41°26'28"N–33°17'65"E, 05.07.2011 (MYP 872). (E)
2. *I. helenium* ssp. *pseudohelenium*, at Kırşehir-Çiçekdağ crossroads, towards the Çiçekdağ, 2. km, Kırşehir, 1070 m, 39°17'21"N–34°07'25"E, 06.09.2010 (MYP 950).
3. *I. helenium* L. ssp. *turcaracemosa* (Grierson), below the Tortum waterfall, Erzurum, 950 m, 40°40'11"N–41°39'62"E, 03.08.2010 (MYP 796).
4. *I. helenium* ssp. *vanensis* (Grierson), north of Çatak, Van, 1695 m, 38°01'15"N–43°02'72"E, 06.08.2010 (MYP 809). (E)

They were identified by senior taxonomist Prof. Dr. Ahmet AKSOY from Akdeniz University, Department of Biology. The voucher specimens were deposited at the Herbarium of the Department of Biology, Erciyes University, Kayseri, Turkey. *I. helenium* ssp. *orgyalis* and *I. helenium* ssp. *vanensis* are endemic (E) to Turkish flora (Davis, 1975).

2.3. Extraction

Dried aerial parts of the plants at room temperature were crushed in a coffee grinder for 2 min. At 15 s intervals, the process was stopped for 15 s to avoid over-heating the sample. Powdered plant samples (10 g) were separately extracted using a Soxhlet type extractor with 100 mL methanol, ethanol, water and ethyl acetate. Thereafter, the extracts were filtered through Whatman No. 1 filter paper and evaporated to dryness in a vacuum at 40°C with a rotary evaporator (Rotavator, Buchi, Switzerland; T<40°C). After determining the yields, the prepared extracts were stored at 4°C until further analysis. The extracts obtained in indicated solvents were expressed as methanol extract (ME), ethanol extract (EE) water extract (WE) and ethyl acetate (EAE), respectively.

2.4. Determination of total phenolics

The total phenolic contents in the plant extracts were estimated by a colorimetric assay based on procedures described by Singleton and Rossi (1965). In short, a 40 µL aliquot of plant extracts dissolved in the same solvent was pipetted into a test tube containing 2.4 mL of distilled water. After mixing the contents, 200 µL of Folin Ciocalteu's phenol reagent and 600 µL of a saturated sodium carbonate solution (20% Na_2CO_3) were added. The contents were vortexed for 15 s and then left to stand at room temperature for 2 h. Absorbance measurements were recorded at 765 nm using a Shimadzu 1240 spectrophotometer and gallic acid was used in the construction of the standard curve. Estimation of the phenolic content was carried out in triplicate. The results were mean values and expressed as mg of gallic acid equivalents (GAE) /g of dry extract.

2.5. Determination of antioxidant activity

2.5.1. Phosphomolybdenum assay

The antioxidant activities of the plant extracts were determined by the phosphomolybdenum method of Prieto et al. (1999). 0.4 mL of the plant extract (1 mg/mL) was mixed with 4 mL of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the green phosphomolybdenum complex was measured at 695 nm. In the case of the blank, 0.4 mL of solvent was used in place of the sample. The antioxidant activity was determined using a standard curve with ascorbic acid solutions as the standard. The mean of three readings was used, and the reducing capacities of the extracts were expressed as mg of ascorbic acid equivalents (AAE)/g extract.

2.5.2. β-Carotene bleaching assay

The extracts ability to inhibit the bleaching of the β-carotene–linoleic acid emulsion was determined

(Cao et al 2009). β-carotene (10 mg) was dissolved in 10 mL of chloroform ($CHCl_3$). An aliquot (0.2 mL) of this solution was added to a boiling flask containing 20 mg of linoleic acid and 200 mg of Tween 40. The chloroform was removed using a rotary evaporator at 40 ^oC for 5 min. Distilled water (50 mL) was slowly added to the residue and mixed vigorously to form an emulsion. The emulsion (5 mL) was added to a tube containing 0.2 mL of the extract solution. The test emulsion was incubated in a water bath at 50 ^oC for 2h, at which point the absorbance was measured at 470 nm. In the negative control, the extract was substituted with an equal volume of ethanol. BHT (Butylated hydroxytoluene) was used as the positive control.

2.5.3. DPPH Assay

The hydrogen atom or electron donation abilities of the plant extracts were measured by bleaching the purple coloured DPPH (2,2-diphenyl-1-picrylhydrazyl) methanol solution. This spectrophotometric assay uses the DPPH stable radical as a reagent (Lee et al 1998). Fifty microliters of various concentrations (0.1-2 mg/mL) of the plant extract in the same solvent were added to 1 mL of 0.1 mM DPPH methanol solution. After a 30 min incubation period at room temperature, absorbance was read against a blank at 517 nm. IC_{50} (the concentration required to scavenge 50% DPPH free radicals) values of the plant extracts were determined graphically. The same procedure was repeated with BHT as a positive control. The measurements were performed in triplicate, and the results were averaged.

Radical scavenging activity was expressed as a percentage inhibition of the DPPH radical and was calculated by the following equation:

Inhibition% = $(A_{blank} - A_{sample} / A_{blank}) \times 100$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound.

2.6. Determination of antimicrobial activity

2.6.1. Microorganisms

The following microroganisms, obtained from the Department of Food Engineering, Erciyes University, Kayseri, Turkey, were used in this study: *Aeromonas hydrophila* ATCC 7965, *Bacillus brevis* FMC 3, *Bacillus cereus* RSKK 863, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 27736, *Listeria monocytogenes* 1/2B**,** *Morganella morganii, Proteus mirabilis* BC 3624, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* NRRLE 4463, *Staphylococcus aureus* ATCC 29213, *Yersinia enterocolitica* ATCC 1501, *Candida albicans* ATCC 1223 and *Saccharomyces cerevisiae* BC 5461.

2.6.2. Agar-well diffusion method

Antimicrobial activity assays of the extracts were carried out using the agar-well diffusion method (Sagdic et al 2009). Each microorganism was suspended in sterile nutrient broth. Test yeasts (*C. albicans*, *S. cerevisiae*) were suspended in malt extract broth. Suspensions of microorganisms, adjusted to 10^6-10^7 colony-forming units (cfu)/mL, were placed in flasks containing 25 mL of sterile nutrient or malt extract agar at 45 ^oC. The mix was poured into Petri plates (9 cm in diameter). The agars were then allowed to solidify at 4 ^oC for 1 h. The wells (5 mm in diameter) were cut from the agar. The extracts were prepared at 1%, 2.5%, 5% and 10% concentrations in the same solvent and 40 µL of the extract solutions were then applied to the wells. The absolute methanol, absolute ethanol, water and ethyl acetate without herb extract were used as a control. *Y. enterocolitica*, *C. albicans* and *S. cerevisiae* were incubated at 25 ^oC for 24-48 h in an inverted position. The other microorganisms were incubated at 37 ^oC for 18-24 h. At the end of the incubation period, all plates were examined for any zones of growth inhibition, and the diameters of these zones were measured in millimeters.

2.7. Statistical analysis

SAS (1988) statistical software was used for data analysis. The comparative analyses between means were conducted using the Tukey multiple range test. Data were subjected to analysis of variance (Two-way ANOVA). Bivariate correlations were analyzed by Pearson's test using SPSS 10.0 (1965) on Windows.

3. Results and discussion

This study determined the antioxidant and antimicrobial activities of the extracts (ME, EE, WE and EAE) of four *I. helenium* taxa growing Turkish flora. The percentage yields of extracts ranged from 1.53 to 27.66 (w/w) (Table 1). Among the extracts of plants, WEs had highest yields while EAEs had lowest yields.

Table 1. The percentage yields of methanol, ethanol, water and ethyl acetate extracts of *I. helenium* taxa (g extract/100 g plant)

Plants	% Yields			
	Methanol Extract	Ethanol Extract	Water Extract	Ethyl acetate Extract
I. helenium ssp. *orygalis*	9.051	7.00	26.39	1.53
I. helenium ssp. *pseudohelenium*	10.44	10.07	20.06	4.75
I. helenium ssp. *turcarasemosa*	14.04	9.60	18.76	4.83
I. helenium ssp. *vanensis*	16.42	11.26	27.66	4.78

The content of total phenolic compounds in the extracts of four *I. helenium* taxa is determined using Folin–Ciocalteau assay and is expressed as gallic acid equivalents (GAE). Statistical differences among the total phenolic contents of the extracts obtained from four *I. helenium* taxa were important ($p < 0.05$). Results in Table 2 showed that the contents of total phenolic compounds in the extracts ranged from 4.18 to 102. 91 mg GAE/g extract. Among the MEs, *I. helenium* ssp. *orygalis* contained the highest amount of total phenolic compounds, while the lowest was in *I. helenium* ssp. *turcarasemosa*. Among the EEs, the highest level of phenolics was found in *I. helenium* ssp *orygalis*, while the lowest was in *I. helenium* ssp. *turcarasemosa*. Among the WEs, the highest level of phenolics was found in *I. helenium* ssp. *vanensis*, while the lowest was in *I. helenium* ssp. *pseudohelenium.* Among the EAEs, the highest level of phenolics was found in *I. helenium* ssp. *orygalis*, while the lowest was in *I. helenium* ssp. *turcarasemosa*. The total phenolic contents of extracts followed the order; ME = EE > WE > EAE.

Table 2. The total phenolic contents of methanol, ethanol, water and ethyl acetate extracts of *I. helenium* taxa

Plants	The total phenolic contents (mg GAE/g extract)*			
	Methanol Extract	Ethanol Extract	Water Extract	Ethyl acetate Extract
I. helenium ssp *orygalis*	$102.91^{Aa} \pm 0.6$	$37.31^{Ab} \pm 0.2$	$17.34^{Bc} \pm 0.3$	$9.60^{Ad} \pm 0.2$
I. helenium ssp. *pseudohelenium*	$23.13^{Ba} \pm 4.4$	$21.44^{Ca} \pm 0.5$	$12.88^{Cb} \pm 0.0$	$6.42^{Bc} \pm 0.4$
I. helenium ssp. *turcarasemosa*	$13.32^{Cb} \pm 1.2$	$17.30^{Da} \pm 0.9$	$17.72^{Ba} \pm 0.1$	$4.18^{Dc} \pm 0.0$
I. helenium ssp. *vanensis*	$24.82^{Ba} \pm 2.3$	$24.64^{Ba} \pm 0.5$	$20.13^{Ab} \pm 0.2$	$5.24^{Cc} \pm 0.0$

*Results are reported as means ± standard deviation (n = 3). Total phenolic content expressed as gallic acid equivalent (GAE).
ABCapital letters in the same column represent the statistical differences between plants tested $p<0.05$,
abLowercase letters in the same row represent the statistical differences between extracts at $p<0.05$.

Total antioxidant capacities of extracts were determined using by phosphomolbydenum assay and are expressed as ascorbic acid equivalents (AAE). Table 3 showed that there were statistical differences among the extracts of four *I. helenium* taxa ($p < 0.05$). Total antioxidant activities of MEs ranged from 120.97–225.80 mg AAE/g extract. *I. helenium* ssp. *orygalis* had the highest total antioxidant activity, while *I. helenium* ssp. *pseudohelenium* had the lowest total antioxidant activity. While *I. helenium* ssp. *vanensis* showed the highest antioxidant activity with 60.91 mg AAE/g extract, *I. helenium* ssp. *pseudohelenium* showed the lowest activity with 33.84 mg AAE/g extract, among the EEs. Total antioxidant activities of WE ranged from 48.10 mg AAE/g extract (*I. helenium* ssp. *orygalis*) to 73.20 mg AAE/g extract (*I. helenium* ssp. *vanensis*). Total antioxidant activities of EAEs ranged from 113.74 mg AAE/g extract (*I. helenium* ssp. *pseudohelenium*) to 307.10 mg AAE/g extract (*I. helenium* ssp. *orygalis*). Among all of the tested extracts, EAE of *I. helenium* ssp. *orygalis* had the highest antioxidant activity, while EE of *I. helenium* ssp. *pseudohelenium* had the lowest antioxidant activity (Table 3).

Table 3. The total antioxidant activities of *I. helenium* taxa

Plants	Antioxidant activity (mg AAE/g extract)*			
	Methanol Extract	Ethanol Extract	Water Extract	Ethyl acetate Extract
I. helenium ssp. *orygalis*	225.80 ^{Ab} ± 0.4	54.10 ^{Bc} ± 0.1	48.10 ^{Cd} ± 0.4	307.10 ^{Aa} ± 0.4
I. helenium ssp. *pseudohelenium*	120.97 ^{Da} ± 0.9	33.84 ^{Dd} ± 0.1	48.97 ^{Cc} ± 0.1	113.74 ^{Db}± 2.4
I. helenium ssp. *turcarasemosa*	183.92 ^{Ba} ± 3.1	47.43 ^{Cd} ± 0.3	62.37 ^{Bc} ± 0.3	149.88 ^{Cb} ± 2.2
I. helenium ssp. *vanensis*	172.32 ^{Cb} ± 1.5	60.91 ^{Ad} ± 0.1	73.20 ^{Ac} ± 0.6	262.29 ^{Ba}± 2.1

*Results are reported as means ± standard deviation (n = 3). Total antioxidant activity expressed as ascorbic acid equivalent (AAE)
^{AB}Capital letters in the same column represent the statistical differences between plants tested p<0.05
^{ab}Lowercase letters in the same row represent the statistical differences between extracts at p<0.05

In the β-carotene/linoleic acid bleaching assay, the oxidation of linoleic acid generates peroxyl free radicals. Thereby, producing free radicals will oxidize the highly unsatured β-carotene resulting in a bleaching effect. The presence of antioxidants in the extract will minimize the oxidation of β-carotene by hydroperoxides. The extract which possesses antioxidant effect may prevent oxidation of lipid components within cell membranes. Therefore, the plant extract may prove to be of potential health benefit (Dastmalchi et al., 2007, Cao et al., 2009; Mariod et al., 2009). The extracts ability to inhibit the bleaching of the β-carotene–linoleic acid emulsion was determined. The presence of the obtained extracts in the linoleic acid emulsion was able to reduce oxidation of β-carotene by hydroperoxides. Table 4 shows inhibition of oxidation in percentage of extracts. All of the extracts exhibited effective antioxidant activity at different degree (p< 0.05). Among the MEs, *I. helenium* ssp. *pseudohelenium* and *I. helenium* ssp. *turcarasemosa* were able to effectively inhibit the oxidation (76.70% and 73.75%, respectively) at concentrations of 1 mg/mL, which was close to the positive control BHT (84.26%) at the same concentration. The effect of *I. helenium* ssp. *pseudohelenium* and *I. helenium* ssp. *turcarasemosa* on the coupled oxidation of linoleic acid and β–carotene was the highest (64.60% and 64.31%, respectively) among the EEs. The inhibition was highest in WE of *I. helenium* ssp. *orygalis* (74.50%), while it is lowest in WE of *I. helenium* ssp. *vanensis* (40.26%). The oxidation of linoleic acid was effectively inhibited by EAE of *I. helenium* ssp. *pseudohelenium* (65.66%), while *I. helenium* ssp. *vanensis* was not able to effectively inhibit the oxidation (38.43%).

Table 4. %Inhibition values of *I. helenium* taxa in β-Carotene - Linoleic acid system

Plants	% Inhibition*			
	Methanol Extract	Ethanol Extract	Water Extract	Ethyl acetate Extract
I. helenium ssp. *orygalis*	61.40 ^{Bb} ± 0.6	56.30 ^{Bc} ± 2.2	74.50 ^{Aa} ± 0.3	55.30 ^{Bc} ± 1.0
I. helenium ssp. *pseudohelenium*	76.70 ^{Aa} ± 1.5	64.60 ^{Ab} ± 1.4	46.03 ^{Bc} ± 0.6	65.66 ^{Ab}± 2.7
I. helenium ssp. *turcarasemosa*	73.75 ^{Aa} ± 1.7	64.31 ^{Ab} ± 1.1	45.36 ^{Bd} ± 2.4	52.01 ^{Bc} ± 1.1
I. helenium ssp. *vanensis*	54.42 ^{Ca} ± 0.1	55.26 ^{Ba} ± 0.7	40.26 ^{Cb} ± 2.3	38.43 ^{Cb} ± 0.5

*Results are reported as means ± standard deviation (n=3)
^{AB}Capital letters in the same column represent the statistical differences between plants tested p<0.05
^{ab}Lowercase letters in the same row represent the statistical differences between extracts at p<0.05

The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The DPPH scavenging activities of different extracts are shown in Table 5. The differences between extracts obtained by various solvent and four *I. helenium* taxa were statistically significant (p<0.05). Free radical scavenging activity also increased with increasing concentration. The scavenging activities of EAEs were much lower than other extracts at all tested concentrations. When compared to the BHT, the scavenging effect of *I. helenium* spp. *orygalis*

extracts and standard on the DPPH radical decreased in the order of BHT>EE>ME>WE and were 92.15%, 92.08%, 91.20% and 90.29% at the dose of 2.0 mg/mL, respectively. Also, the percentage inhibition value of ME obtained from *I. helenium* spp. *pseudohelenium* (91.93%), WE obtained from *I. helenium* spp. *turcarasemosa* (92.19%), ME and WE obtained from *I. helenium* spp. *vanensis* (91.88% and 92.09%, respectively) were close to that of BHT at the same concentration. These results indicated that all extracts except EAE have a noticeable effect on scavenging free radicals.

Table 5. % Inhibition values of *I. helenium* taxa in DPPH free radical scavenging assay

Con. (mg/mL)	Extract	*I. helenium* ssp. *orygalis*	*I. helenium* ssp. *pseudohelenium*	*I. helenium* ssp. *turcarasemosa*	*I. helenium* ssp. *vanensis*
0.1	ME	16.28 ^{Aa} ± 0.4	6.40 ^{Ab} ± 0.1	4.97 ^{Bc} ± 0.2	6.46 ^{Ab} ± 0.0
	EE	10.10 ^{Ba} ± 0.4	5.69 ^{Bb} ± 0.2	2.24 ^{Cc} ± 0.2	1.19 ^{Cd} ± 0.2
	WE	8.43 ^{Ba} ± 1.3	2.29 ^{Db} ± 0.2	7.55 ^{Aa} ± 0.1	6.81 ^{Aa} ± 0.2
	EAE	0.22 ^{Cb} ± 0.0	2.82 ^{Ca} ± 0.2	- ^{Db} ± 0.0	2.88 ^{Ba}± 0.0
0.25	ME	47.04 ^{Aa} ± 0.2	14.71 ^{Ac} ± 0.1	12.64 ^{Bd} ± 0.1	15.23 ^{Bb} ± 0.1
	EE	33.67 ^{Bb} ± 0.6	13.34 ^{Bb} ± 0.1	6.90 ^{Cc} ± 0.1	10.92 ^{Cc} ± 0.2
	WE	15.82 ^{Cab} ± 0.6	6.32 ^{Cc}± 0.2	15.74 ^{Ab} ± 0.2	16.78 ^{Aa} ± 0.1
	EAE	7.17 ^{Da} ± 0.1	5.65 ^{Db} ± 0.2	0.96 ^{Dc} ± 0.4	5.31 ^{Db} ± 0.3
0.5	ME	88.45 ^{Aa} ± 0.5	28.08 ^{Ac} ± 0.1	23.93 ^{Bd} ± 0.1	29.15 ^{Bb} ± 0.1
	EE	67.69 ^{Ba} ± 0.1	24.77 ^{Bb} ± 0.3	14.67 ^{Cd} ± 0.1	22.06 ^{Cc} ± 0.1
	WE	26.85 ^{Cc} ± 0.6	16.67 ^{Cd} ± 0.2	30.63 ^{Ab} ± 0.3	32.77 ^{Aa} ± 0.1
	EAE	15.64 ^{Da} ± 0.1	9.11 ^{Db} ± 0.1	3.43 ^{Dd} ± 0.4	7.69 ^{Dc} ± 0.4
1.0	ME	90.19 ^{Aa} ± 0.4	54.36 ^{Ac} ± 0.3	46.51 ^{Bd} ± 0.2	56.76 ^{Bb} ± 0.1
	EE	90.19 ^{Aa} ± 0.3	49.07 ^{Bb} ± 0.3	29.34 ^{Cd} ± 0.1	43.99 ^{Cc} ± 0.3
	WE	50.51 ^{Bc} ± 0.4	33.24 ^{Cd} ± 0.1	58.17 ^{Ab} ± 0.3	62.91 ^{Aa} ± 0.3
	EAE	22.22 ^{Ca} ± 0.4	15.66 ^{Db} ± 0.1	4.96 ^{Dd}± 0.6	11.82 ^{Dc} ± 0.8
2.0	ME	91.20 ^{Bb} ± 0.0	91.93 ^{Aa} ± 0.0	80.30 ^{Bc} ± 0.4	91.88 ^{Aa} ± 0.1
	EE	92.08 ^{Aa} ± 0.0	87.74 ^{Bb} ± 0.1	53.57 ^{Cd} ± 0.4	81.26 ^{Bc} ± 0.3
	WE	90.29 ^{Cb} ± 0.5	64.37 ^{Cc} ± 0.2	92.19 ^{Aa} ± 0.1	92.09 ^{Aa} ± 0.1
	EAE	28.36 ^{Db} ± 0.2	30.25 ^{Da} ± 0.6	7.91 ^{Dd} ± 0.1	20.43 ^{Cc} ± 0.5

^{AB}Capital letters in the same column represent the statistical differences between extracts $p < 0.05$
^{ab}Lowercase letters in the same row represent the statistical differences between plants tested at $p < 0.05$

The concentrations providing 50% inhibition (IC_{50}) are given in Table 6. These tables indicate that the four *I. helenium* taxa and their extracts possess potent free radical scavenging activity. The results revealed that MEs had the highest effective radical scavenging activity, followed by WEs and EAs. The lowest activities were found in the EAEs. The IC_{50} value for EAE of *I. helenium* ssp. *turcarasemosa* was not determined because of low activity at tested concentrations. ME of *I. helenium* ssp. *orygalis* was found to have the highest radical scavenging activity (IC_{50}= 8.89 µg/mL). Lower IC_{50} value indicates better antiradical activity.

Correlations between phenolic contents and antioxidant activities were investigated. There was not a substantial correlation between the phenolic contents versus total antioxidant activities (r^2= 0.119), antioxidant activity as inhibition of β-carotene bleaching (r^2= 0.133). It was observed that there is correlation between the free radical scavenging activities versus the total antioxidant activities (r^2=0,525) and phenolic contents (r^2= -0.404) at 0.01 level.

Table 6. IC_{50} values of *I. helenium* taxa in DPPH assay

Plants	IC_{50} (µg/mL)			
	Methanol Extract	Ethanol Extract	Water Extract	Ethyl acetate Extract
I. helenium ssp. *orygalis*	8.89 ^{Dd}	12.28 ^{Dc}	32.48 ^{Bb}	131.00 ^{Ba}
I. helenium ssp. *pseudohelenium*	30.53 ^{Bd}	34.10 ^{Cc}	51.23 ^{Ab}	111.76 ^{Ca}
I. helenium ssp. *turcarasemosa*	36.76 ^{Ab}	61.70 ^{Aa}	28.35 ^{Cc}	- ^{Dd}
I. helenium ssp. *vanensis*	29.21 ^{Cc}	38.67 ^{Bb}	26.15 ^{Dd}	198.16 ^{Aa}

-Not determined
^{AB}Capital letters in the same column represent the statistical differences between plants tested $p<0.05$
^{ab}Lowercase letters in the same row represent the statistical differences between extracts at $p<0.05$

The antibacterial activities of the extracts of four *I. helenium* taxa were determined against eight Gram (-), five Gram (+) bacteria and two yeasts as shown in Table 7-10. The data obtained from the antimicrobial assay indicated that the extracts of four *I. helenium* taxa displayed a variable degree of antimicrobial activity on different tested strains. The inhibitory effect increased with increase of the extract concentration from 1-10%. The solvents used for control did not show any activity (results not shown). ME of *I. helenium* spp. *orygalis* inhibited all Gram (-) bacteria except *S. typhimurium*. ME of *I. helenium* spp. *orygalis* inhibited all Gram (+) bacteria except *B. brevis* and *B. subtilis*. EE of *I. helenium* spp. *orygalis* inhibited all Gram (-) bacteria except *A. hydrophila*. EE of *I. helenium* spp. *orygalis* inhibited all Gram (+) bacteria except *B. brevis*. EAE of *I. helenium* spp. *orygalis* inhibited all Gram (-) bacteria except *A. hydrophila* and *Y. enterocolitica.* EAE of *I. helenium* spp. *orygalis* inhibited all Gram (+) bacteria except *B. brevis*. WE of *I. helenium* spp. *orygalis* inhibited only *P. aeruginosa*, *B. cereus* and *L. monocytogenes*. All extracts had no effect against yeasts tested. *C. albicans* was only inhibited by EE of *I. helenium* spp. *orygalis. P. aeruginosa* was the most sensitive a Gram (-) bacterium for all extracts of *I. helenium* spp. *orygalis*, while *B. cereus* was the most sensitive a Gram (+) bacterium (Table 7). The data from Table 8 showed that *E. coli*, *M. morganii* and *B. subtilis* were only inhibited by EE of *I. helenium* spp. *pseudohelenium*. *Y. enterocolitica* was only inhibited by ME of *I. helenium* spp. *pseudohelenium* at 10%. WE of *I. helenium* spp. *pseudohelenium* had no effects against all bacteria tested. All extracts of *I. helenium* spp. *pseudohelenium* had no inhibitory effects against yeasts tested. *P. mirabilis* was not inhibited by any of the extracts tested of *I. helenium* spp. *pseudohelenium*.

Table 7. Antimicrobial activities of *I. helenium* ssp. *orygalis*

Microorganisms	Extracts (% Concentrations)															
	ME				EE				WE				EAE			
	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1
Gram (-)																
A. hydrophila	11.0*	10.0	9.0	8.0	-	-	-	-	-	-	-	-	-	-	-	-
E. coli	7.0	-	-	-	8.0	7.0	-	-	-	-	-	-	7.0	-	-	-
M. morganii	8.0	7.0	-	-	9.0	8.0	-	-	-	-	-	-	7.0	-	-	-
K. pneumoniae	10.0	8.0	-	-	8.0	7.0	-	-	-	-	-	-	9.0	7.0	-	-
P. mirabilis	7.0	-	-	-	9.0	7.0	-	-	-	-	-	-	7.0	-	-	-
P. aeruginosa	22.0	20.0	15.0	8.0	18.0	16.0	10.0	-	12.0	10.0	8.0	-	23.0	20.0	22.0	21.0
S. typhimurium	-	-	-	-	7.5	7.0	-	-	-	-	-	-	8.0	7.0	-	-
Y. enterocolitica	8.0	0.0	0.0	0.0	8.0	7.0	-	-	-	-	-	-	-	-	-	-
Gram (+)																
B. brevis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus	28.0	22.0	20.0	16.0	28.0	25.0	20.0	18.0	20.0	16.0	10.0	7.0	25.0	22.0	22.0	11.0
B. subtilis	-	-	-	-	8.0	7.0	-	-	-	-	-	-	9.0	7.0	-	-
L. monocytogenes	24.0	20.0	15.0	8.0	25.0	21.0	18.0	10.0	16.0	10.0	7.0	-	22.0	22.0	18.0	8.0
S. aureus	16.0	11.0.	10.0	-	15.0	11.0	8.0	-	-	-	-	-	15.0	7.0	-	-
Yeasts																
C. albicans	-	-	-	-	8.0	7.0	-	-	-	-	-	-	-	-	-	-
S. cerevisiae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*Inhibition zones include diameter of hole (5 mm), sample amount 40 µL.
ME: Methanol extract, EE: Ethanol extract, WE: Water extract, EAE: Ethyl acetate extract
-Not active

Table 8. Antimicrobial activities of *I. helenium* ssp. *pseudohelenium*

Microorganisms	Extracts (% Concentrations)															
	ME				EE				WE				EAE			
	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1
Gram (-)																
A. hydrophila	8.5*	7.5	7.0	-	9.0	8.0	7.0	-	-	-	-	-	8.0	7.0	-	-
E. coli	-	-	-	-	8.0	7.0	-	-	-	-	-	-	-	-	-	-
M. morganii	-	-	-	-	8.0	7.5	7.0	-	-	-	-	-	-	-	-	-
K. pneumoniae	10.0	7.0	-	-	9.0	8.0	7.5	-	-	-	-	-	7.5	7.0	-	-
P. mirabilis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	10.0	9.0	8.5	8	12.0	9.0	8.0	7.0	-	-	-	-	9.0	8.0	-	-
S. typhimurium	-	-	-	-	8.0	7.0	-	-	-	-	-	-	8.0	7.0	6.5	-
Y. enterocolitica	8.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gram (+)																
B. brevis	11.0	8.0	-	-	10.0	11.0	-	-	-	-	-	-	-	-	-	-
B. cereus	12.0	8.0	-	-	11.0	8.0	6.5	-	-	-	-	-	8.0	7.5	7.0	-
B. subtilis	-	-	-	-	8.0	7.0	-	-	-	-	-	-	-	-	-	-
L. monocytogenes	11.0	8.0	-	-	9.0	8.0	7.5	-	-	-	-	-	10.0	8.0	-	-
S. aureus	11.0	8.0	7.5	-	9.0	8.0	-	-	-	-	-	-	10.0	8.0	-	-
Yeasts																
C. albicans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*Inhibition zones include diameter of hole (5 mm), sample amount 40 µL.
ME: Methanol extract, EE: Ethanol extract, WE: Water extract, EAE: Ethyl acetate extract
-Not active

Results from the antimicrobial activity assay of *I. helenium* spp. *turcarasemosa* are summarized in Table 9. WE of *I. helenium* spp. *turcarasemosa* had no effects against all bacteria tested. All extracts of *I. helenium* spp. *turcarasemosa* had no inhibitory effects against yeasts tested. *S. typhimurium* was only inhibited by EE, whereas *B. brevis* was only inhibited by ME of *I. helenium* spp. *turcarasemosa*. According to results given in Table 10, ME, EE and EAE of *I. helenium* spp. *vanensis* showed antibacterial activity against all Gram (-) bacteria tested. ME of *I. helenium* spp. *vanensis* had no effect against *B. brevis*, whereas EAE of *I. helenium* spp. *vanensis* had no effect against *B. cereus* among the Gram (+) bacteria tested. WE of *I. helenium* spp. *vanensis* had no effects against all bacteria tested. All extracts of *I. helenium* spp. *vanensis* had no inhibitory effects against yeasts tested.

Table 9. Antimicrobial activities of *I. helenium* ssp. *turcarasemosa*

Microorganisms	Extracts (% Concentrations)															
	ME				EE				WE				EAE			
	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1
Gram (-)																
A. hydrophila	8.0*	7.5	7.0	-	7.5	7.0	-	-	-	-	-	-	10.0	8.0	7.0	-
E. coli	7.5	-	-	-	7.5	7.0	6.5	6.0	-	-	-	-	10.0	8.0	7.0	-
M. morganii	-	-	-	-	-	-	-	-	-	-	-	-	9.0	8.0	-	-
K. pneumoniae	8.0	7.5	7.5	7.0	9.0	8.5	8.0	7.0	-	-	-	-	8.0	7.0	-	-
P. mirabilis	7.0	-	-	-	-	-	-	-	-	-	-	-	8.0	-	-	-
P. aeruginosa	9.0	-	8.0	-	11.0	10.0	9.0	8.0	-	-	-	-	9.5	8.5	7.0	6.5
S. typhimurium	-	-	-	-	7.5	7.0	6.5	6.0	-	-	-	-	-	-	-	-
Y. enterocolitica	8.0	-	-	-	8.0	-	-	-	-	-	-	-	10.0	8.0	7.0	-
Gram (+)																
B. brevis	8.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B. cereus	-	-	-	-	18.0	16.0	15.0	8.0	-	-	-	-	9.0	8.0	7.0	-
B. subtilis	-	-	-	-	7.0	7.0	6.5	6.0	-	-	-	-	7.0	7.5	8.0	7.0
L. monocytogenes	11.0	-	-	-	9.0	8.0	7.0	6.5	-	-	-	-	10.0	8.0	-	-
S. aureus	8.0	7.5	7.0	-	10.0	7.5	7.0	-	-	-	-	-	10.0	8.0	-	-
Yeasts																
C. albicans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*Inhibition zones include diameter of hole (5 mm), sample amount 40 µL.
ME: Methanol extract, EE: Ethanol extract, WE: Water extract, EAE: Ethyl acetate extract
-Not active

Table 10. Antimicrobial activities of *I. helenium* ssp. *vanensis*

Microorganisms	Extracts (% Concentrations)															
	ME				EE				WE				EAE			
	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1	10	5	2.5	1
Gram (-)																
A. hydrophila	8.0*	7.0	6.5	6.0	9.0	8.0	6.5	-	-	-	-	-	12.0	11.0	7.0	-
E. coli	6.5	6.5	6.0	6.0	9.0	8.0	7.0	-	-	-	-	-	9.0	8.0	7.5	7.0
M. morganii	6.5	6.0	-	-	8.0	7.0	6.5	-	-	-	-	-	10.0	8.0	7.0	-
K. pneumoniae	8.0	7.0	6.5	-	10.0	9.0	8.0	6.0	-	-	-	-	11.0	9.0	-	-
P. mirabilis	8.0	7.0	6.5	6.0	10.0	8.0	7.5	7.0	-	-	-	-	9.0	8.0	7.0	6.5
P. aeruginosa	10.0	8.0	7.5	6.5	11.0	9.0	8.0	7.0	-	-	-	-	12.0	-	-	-
S. typhimurium	8.0	7.0	6.0	-	8.0	7.0	-	-	-	-	-	-	8.0	7.5	7.0	-
Y. enterocolitica	8.0	7.0	-	-	10.0	8.0	-	-	-	-	-	-	10.0	7.0	-	-
Gram (+)																
B. brevis	-	-	-	-	14.0	10.0	6.5	-	-	-	-	-	11.0	10	8	-
B. cereus	7.5	7.0	6.5	6.0	17.0	15.0	11.0	7.0	-	-	-	-	-	-	-	-
B. subtilis	8.0	7.0	-	-	9.0	8.0	7.0	6.5	-	-	-	-	8.0	7.5	7.5	7.0
L. monocytogenes	8.0	7.0	7.0	6.0	10.0	8.0	7.0	-	-	-	-	-	13.0	12.0	8.0	-
S. aureus	8.0	7.0	6.0	-	9.0	7.0	-	-	-	-	-	-	11.0	10.0	6.5	-
Yeasts																
C. albicans	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
S. cerevisiae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*Inhibition zones include diameter of hole (5 mm), sample amount 40 µL
ME: Methanol extract, EE: Ethanol extract, WE: Water extract, EAE: Ethyl acetate extract
-Not active

According to our literature survey, there are only four studies on the *Inula* species growing to Turkish flora (Karamenderes and Zeybek, 2000; Akay, 2002; Gençaslan, 2007; Sengul et al., 2009). Karamenderes and Zeybek (2000) reported the chemical compositions of essential oils of *I. viscosa*, *I. graveolens* and *I. helenium* ssp. *turcarasemosa*. In another study, essential oils of *I. heterolepis* obtained by hydrodistillation and steam distillation were analyzed and 34 and 32 compounds were identified, respectively (Akay, 2002). The main components of essential oils obtained by hydrodistillation were determined as Borneol (%50.3) and Exobornyl acetate (%39.99). Sengul et al. (2009) determined total phenolic content of the methanol extract obtained from *I. aucherana* as 6.57 mg GAE/g. This value is much lower than that of methanol extracts obtained from *I. helenium* taxa (13.32-102.91 mg GAE/g extract) tested in our study. The IC_{50} values of methanol and ethyl acetate extracts of *I. britannica* were determined as 0.033 mg/mL, 0.19 mg/mL, respectively in DPPH assay (Gençaslan, 2007).

Total phenolic contents of methanol and water extracts of *I. viscose* were found as 7.5±0.4 ve 4.0±0.7 mg GAE/g and these extracts were reported to possess low antioxidant activity (Al- Mustafa and Al-Thunibat, 2008). Total phenolic contents of four *I. helenium* taxa in methanol and water extracts were much higher than that of *I. viscose.* Wojdyło et al. (2007) reported that the total phenolic content and DPPH radical scavenging activity of *I. helenium* was 3.65±0.12 µM trolox equivalent/100 g dry weight and 144±1.04 µM troloks/100 g weight, respectively.

Eight flavonoids (luteolin, diosmetin, chrysoeriol, kaempferol, quercetin, 6- hydroxyluteolin-6-methyl ether, spinacetin and eupatin) were identified from the flowers of *I. britannica* var. *chinensis* by Bai et al. (2005) and their antioxidant activities were shown. It has been reported that flowers or the aerial parts of *I. britannica* are a rich source of secondary metabolites such as terpenoids (sesquiterpene lactones and dimmers, diterpenes and triterpenoids), and flavonoids and they show diverse biological activities: anticancer, antioxidant, anti-inflammatory, neuroprotective and hepatoprotective activities (Khan et al., 2010).

Similar to our study, antibacterial activities of ethyl acetate extract of *I. racemosa* roots against *P. aeruginosa* (12 mm), *B. cereus* (9 mm), *E. coli* (14 mm), *Salmonella typhi* (15 mm), *S. aureus* (10 mm) and *K. pneumoniae* (15 mm) were determined by Lokhande et al. (2007). It was determined that the methanol extract of *I. aucherana* had inhibitory effects against *P. aeruginosa* (10 mm), *S. aureus* (15 mm), *Paecilomyces variorti* (10 mm) and *B. cereus* (15 mm) in the disc diffusion test (Sengul et al., 2009). In another study, it was shown that essential oil and extracts of *I. helenium* roots had clear activity against *B. cereus*, *S. aureus* and *Enterococcus faecium*, and *P. aeruginosa* was the least susceptible to *I. helenium* oil (Deriu et al., 2008). Zhao et al. (2010) showed that the thymol

derivatives isolated from the roots of *I. hupehensis* had antimicrobial activities against three bacteria (*S. aureus*, methicillin-resistant *S. aureus* and *E. coli*) and three plant pathogenic fungi (*Rhizoctonia solani*, *Phytophthora melonis* and *Peronophythora litchi*). Similarly, 10-Isobutyryloxy-8,9-epoxythymol isobutyrate from *I. helenium ve I. royleana* have been proven to possess antibacterial activities against *S. aureus* and *E. coli* (Stojakowska et al. 2005). It is difficult to compare the results of different studies due to different species, different solvent and method used for the extraction, and/or different methods used for the evaluation of biological activities.

4. Conclusion

The results of the present study showed that the extracts of four *I. helenium* taxa, of which two are endemic, contain high amount of phenolic compounds and exhibited the clear antioxidant and antimicrobial activity. Total phenolic contents, antioxidant and antimicrobial activities of *I. helenium* ssp. *turcarasemosa*, *I. helenium* ssp. *vanensis*, *I. helenium* ssp. *pseudohelenium*, *I. helenium* ssp. *orygalis* growing in Turkey have been reported here for the first time. Hence, *I. helenium* taxa could be a good source of antioxidant and antimicrobial agents in foods, pharmaceuticals and cosmetic preparations. Further studies are warranted for the isolation and also *in vivo* studies are need for better understanding their mechanism of action as antioxidant and antimicrobial agents.

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