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Variation in antioxidant, and antibacterial activities and total phenolic content of the bulbs of mooseer (*Allium hirtifolium* Boiss.)

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ABSTRACT

Allium hirtifolium Boiss. (mooseer) belonging to the family Alliaceae, is an endemic species of Iran which grows wild in the Zagros Mountains range, western and southwestern Iran. The bulb of *A. hirtifolium* has been used as a flavouring agent, especially dairy foods and pickles by the indigenous people, southwestern Iran. In this study, the bulbs of various populations of the plant were collected from the alpine regions in Chaharmahal va Bakhtiari province, Iran. The total phenolic content of the ethanol extract was determined by Folin–Ciocalteu method, the antioxidant activity was evaluated by measuring 1,1-diphenyl-2-picrylhydrazyl (DPPH), and the antibacterial activity of the extracts against four bacteria, including *Bacillus cereus*, *Listeria monocytogenes*, *Proteus vulgaris*, and *Salmonella typhimurium* was determined by serial dilution assay. Results indicated that the total phenolic content in the ethanol extracts of different populations of *A. hirtifolium* ranged between 34 to 44 mg gallic acid/g extract. In addition, the extracts of *A. hirtifolium* indicated moderate–to–good inhibitory activities (MICs = 0.062 to 0.250 mg/ml) against four bacteria, especially against *B. cereus*. The antioxidant activity of the bulbs of *A. hirtifolium* indicated the extract acted as an effective DPPH scavenger, but were not as effective as the BHT control. This finding suggests that the bulbs of *A. hirtifolium* may be considered as a natural source of antioxidants and antimicrobial agents.

Key words: Alliaceae, biological activity; endemic herbs; mooseer

IZVLEČEK

SPREMENLJIVOST ANTIOKSIDACIJSKEGA IN ANTIBAKTERIJSKEGA DELOVANJA CELOKUPNIH FENOLNIH IZVLEČKOV IZ ČEBULIC PERZIJSKE ŠALOTKE (*Allium hirtifolium* Boiss.)

Perzijska šalotka (*Allium hirtifolium* Boiss. (mooseer), *Allium stipitatum* Regel) spada v družino lukovk (Alliaceae), je endemična vrsta Irana, ki raste samoniklo v zahodnem in jugozahodnem delu države na območju gorovja Zagros. Prebivalci jugozahodnega Irana jo uporablja kot začimbo v mlečnih izdelkih in vlaganju zelenjave. V tej raziskavi so bile analizirane čebulice različnih populacij, nabrane v alpinskih predelih province Chaharmahal va Bakhtiari. Celokupna vsebnost fenolov je bila določena v etanolnem izvlečku po metodi Folin–Ciocalteu, antioksidativno delovanje je bilo ovrednoteno in izmerjeno z 1,1-difenil-2-pikrilhidrazilom (DPPH), antibakterijsko delovanje izvlečkov je bilo določeno proti štirim vrstam bakterij, *Bacillus cereus*, *Listeria monocytogenes*, *Proteus vulgaris*, in *Salmonella typhimurium* s serijskim razredčitvenim testom. Rezultati so pokazali, da so etanolni izvlečki celokupnih fenolov iz čebulic različnih populacij te vrste vsebovali od 34 do 44 mg galične kisline na g izvlečka. Izvlečki so pokazali zmerno do dobro inhibitorno aktivnost (MICs = 0.062 do 0.250 mg/ml) proti omenjenim štirim vrstam bakterij, še posebej proti vrsti *B. cereus*. Antioksidativno delovanje izvlečkov čebulic je pokazalo, da so izvlečki delovali kot učinkoviti lovilci DPPH, vendar so bili manj učinkoviti kot BHT v kontroli. Izsledki kažejo, da so lahko čebulice perzijske šalotke (*A. hirtifolium*) dober naravni vir antioksidantov in antimikrobnih snovi.

Ključne besede: *A. hirtifolium,* Alliaceae, biološka aktivnost, antioksidant, antimikrobna snov

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1 INTRODUCTION

Plant extracts are rich sources of natural antioxidant and antibacterial compounds. Phenolic compounds present in spice plants as dietary sources possess bioactive properties protecting cellular systems against oxidative stress (Ghasemi Pirbalouti et al., 2013a). Recently, interest in finding naturally occurring antioxidants to replace synthetic antioxidants in foods and medicines has increased considerably, primarily due to the possible carcinogenicity of the synthetic antioxidants (Velioglu et al., 1998).

The genus of *Allium* L. is the largest and important representative genus of the Alliaceae family comprises 700 species; each with different tastes, forms and colors; nonetheless, they are close in biochemical, phytochemical, and nutraceutical properties (Tepe et al., 2005). *Allium* species are revered to possess antibacterial, antifungal, antiviral, antiprotozoal, and anthelmintic activities (Ariga and Seki, 2006; Benkeblia, 2005) and they contain the powerful antioxidants, sulphur and other numerous phenolic compounds which have aroused great interests for food industries. The *Allium* species have been used for a long time as a medicinal for the prevention and treatment of certain diseases such as diabetes, arthritis, colds and flu, stress, fever, coughs, headache, hemorrhoids, asthma, arteriosclerosis, cancer, respiratory, gastrointestinal, rheumatic, and inflammatory disorders (Najjaa, et al., 2009; Kojuri et al., 2007; Amin, 1991). Biological and medical functions of *Allium* species are due to their sulphur compounds, such as S-alk (en) yl-L'Cysteine sulfoxides (Fritsch and Keusgen, 2006), however, presence of phenolic compounds are also beneficial for human health (Corzo-Martinez et al., 2007).

Mooseer (*Allium hirtifolium* Boiss.), is an endemic plant of Iran which wild grows in the alpine regions in Zagros Mountains range from Northwestern to Southwestern of Iran with the climate of very to moderate cold (Ghahreman, 1984; Rechinger, 1984). *A. hirtifolium* is a nutritive plant with special taste which its dried bulb slices are used as an additive to yogurt and also pickling mixtures, rice, meat, sauces and salads. The bulbs of *A. hirtifolium* have been used as a flavouring agent, especially dairy foods and pickles by the indigenous people, southwestern Iran (Ghasemi Pirbalouti, 2009). In Iranian folk medicine, mooseer has been successfully used for treating rheumatic and inflammatory disorders. In addition, different medicinal properties such as antitrichomonas, antiproliferative, and immunomodulatory activities have also been reported for the bulbs of mooseer (Mozaffarian, 2008; Ghodrati Azadi et al., 2008; Jafarian et al., 2003; Amin, 1991). Results of previous studies (Ashrafi et al., 2004; Ismail et al., 2013) indicated that the aqueous and methanol extracts of mooseer bulbs have antimicrobial properties.

Mooseer (*Allium hirtifolium* Boiss.), is an endemic plant of Iran which wild grows in the alpine regions in Zagros Mountains range from Northwestern to Southwestern of Iran with the climate of very to moderate cold (Ghahreman, 1984; Rechinger, 1984). *A. hirtifolium* is a nutritive plant with special taste which its dried bulb slices are used as an additive to yogurt and also pickling mixtures, rice, meat, sauces and salads. The bulbs of *A. hirtifolium* have been used as a flavouring agent, especially dairy foods and pickles by the indigenous people, southwestern Iran (Ghasemi Pirbalouti, 2009). In Iranian folk medicine, mooseer has been successfully used for treating rheumatic and inflammatory disorders. In addition, different medicinal properties such as antitrichomonas, antiproliferative, and immunomodulatory activities have also been reported for the bulbs of mooseer (Mozaffarian, 2008; Ghodrati Azadi et al., 2008; Jafarian et al., 2003; Amin, 1991). Results of previous studies (Ashrafi et al., 2004; Ismail et al., 2013) indicated that the aqueous and methanol extracts of mooseer bulbs have antimicrobial properties.

To our knowledge, there are no published reports on diversity of total phenolic content, antibacterial and antioxidant activities of various populations of *A. hirtifolium.* The main objective of this study was to evaluate content of phenolic compounds, antioxidants and antibacterial activities of the ethanol extracts from the bulbs of various populations of *A. hirtifolium*, and to evaluate them as potential sources of natural antioxidants and antimicrobial.

2 MATERIAL AND METHODS

2.1 Plant material

The samples of the bulb of *A. hirtifolium* collected from wild populations of the plants growing in various alpine regions of southwestern Iran were used in this study. In total, three replicate samples of 30 plants were gathered from three natural habitats at the early flowering between April 30th to May 20th 2012. The slope and elevation information were obtained from the Digital Elevation Model (DEM) using two well-known GIS software packages ILWIS (3.0 Academic). This array was geo-referenced using a metric UTM coordinate system and the geometric correction were carried out in the GIS ILWIS (Table 1). Soil physical and chemical characteristics, including pH, electrical conductivity (EC), organic carbon (OC%), and soil texture were determined (Table 1). Climatic data of the locations were determined using data collected by the nearest meteorology stations (Table 1). Plant identity was confirmed by Prof. V. Mozaffarian, and a representative voucher specimen (No. 1265) was been placed in the Herbarium of Research Center of Natural Resources of Chaharmahal va Bakhtiari province, Shahrekord, Iran.

2.2 Extract preparation

Immediately following collection, the leaves of *A. hirtifolium* from each plant sample were separated and bagged independently. The bulbs were cleaned with tap water and cut into small slices by using a kitchen mixer. The tissue samples were subsequently air-dried in a shaded room at 30 ± 5 °C. A 100 g sample was extracted with 250 ml ethanol (96%, Merck, Darmstadt, Germany) at 45 °C for 8 h followed by a Soxhlet apparatus. The ethanol was subsequently removed under reduced pressure on a rotary evaporator (Model Zirbus 302 W, Italy) at 40 °C. The extracts were filtered using a Whatman No. 2. The extract samples were stored in universal bottles and refrigerated at 4 °C prior to use.

Table 1. Geographical and climate of natural habitats of *Allium hirtifolium*

Region	Altitude (m)	Latitude (UTM)	Longitude (UTM)	P* (mm)	T (°C)	pH	E.C. (dS/m)	O.C (%)	Sand (%)	Silt (%)	Clay (%)
Samsami	2742	0435278	3565206	779.9	12.6	6.85	0.528	1.931	26	36	38
Khaki	2487	0448579	3587078	327.3	10.6	7.5	0.442	1.541	20	42	38
Dasht-e-Laleh	2336	0428599	3599942	1025.1	9.7	7.23	0.348	1.117	32	32	36

* P: Annual precipitation (mm), T: Average temperature (°C), E.C.: Electrical conductivity (dS/m), O.C.: Organic carbon (%).

Meteorological information was obtained from weather stations located within the study area and the surrounding zone; each value in the mean of 10 to 15 year data.

Soil characteristics are based on average of samples taken from three farms in each region.

2.3 Determination of total phenolic content (TPC)

The total amount of phenolic compounds in each extract was determined using the Folin–Ciocalteu method following procedure of Singleton and Rossi (1965) with some modifications. Briefly, 0.5 ml of the sample was mixed with 2.5 ml of Folin- Ciocalteu's (Sigma–Aldrich Co., Steineheim, Germany) phenol reagent for 5 min at 37 °C, 2 ml of saturated Na_2CO_3 (7.5%) (Merck Co., Darmstadt, Germany) was added, and the mixture was brought to 10 ml with the addition of deionized, distilled water. The mixture was maintained at room temperature in the dark for 120 min and then the absorbance was measured at 765 nm against a reagent blank using a Perkin–Elmer Lambda UV/Vis spectrophotometer. Gallic acid (Merck Co., Darmstadt, Germany) was used as the reference standard and the total phenolic content was expressed as mg of gallic acid equivalents per gram of each extract on dry basis (mg GAE/g extract).

2.4 Antioxidant test

The DPPH radical scavenging activity of the ethanol extract was determined using the method proposed by Hung et al. (2005). The extracts

(100 µL) at concentrations of 8, 16, 32, 62.5, 125, 250, and 500 µg/ml were mixed with 3.9 mL an equal volume of 0.2 mM ethanol solution of DPPH (Sigma–Aldrich Co., Steineheim, Germany). The disappearance of the DPPH after 30 min of incubation at room temperature was determined using a Perkin–Elmer Lambda UV/Vis spectrophotometer at 515 nm against a blank, i.e. without DPPH. Ethanol was used to zero the spectrophotometer and the absorbance of the DPPH radical without antioxidant and measure daily served as the control. The amount of sample necessary to decrease the absorbance of DPPH by 50 % (IC_{50}) was calculated graphically and the percentage inhibition was determined according to the equation:

$$\% \text{ inhibition} = \left[\frac{AC(0) - AA(t)}{AC(0)}\right] * 100$$

where AC_0 is the absorbance of the control at t = 0 min and AA_t is the absorbance of the antioxidant at t = 30 min. The food preservative butylated hydroxytoluene (BHT) was used as positive control. All measurements were replicated three times.

2.5 Antibacterial test

Antibacterial activity of the extracts were tested using clinical isolates of four bacteria strains, the Gram-positive bacteria (*Bacillus cereus* and *Listeria monocytogenes*) and the Gram-negative bacteria (*Proteus vulgaris* and *Salmonella typhimurium*). The bacteria, originally obtained from chicken meat samples, were provided by the Food Microbiology Laboratory, Veterinary Medicine Faculty, (I.A.U.) Iran and had been positively identified using PCR-RFLP along with conventional morphological and biochemical tests. The population of each bacterial strain was increased by culturing in an overnight Mueller Hinton broth (MHB) at 37 °C. To quantify the antibacterial activity of the extracts, bacteria populations were prepared for testing by adjusting each population to 1.0 McFarland standards (1.0×10^7 CFU/mL), using a spectrophotometer (Perkin–Elmer Lambda UV/Vis, USA). Minimum inhibitory concentrations (MIC) were determined using the broth-serial dilution method following standardized methods (CLSI, 2012). The extracts and the antimicrobial agents (ciprofloxacin, and flumequine) were each dissolved in 5 % dimethyl sulfoxide (DMSO) and then diluted to the highest test concentration (500 µg/mL). Subsequent test concentrations were made in a series of two-fold dilutions to develop concentration levels of 8 to 500 µg/ml in sterile, 10 ml test tubes containing MHB. A population of bacteria was subsequently added to each tube containing an essential oil or antimicrobial agent and then incubated at 37 °C for 48 h. After the incubation period, the absorbance of each incubated solution was measured at 630 nm using a spectrophotometer (Perkin–Elmer Lambda UV/Vis, USA) as a measure of bacterial growth to indicate MIC values. The minimum bactericidal concentration (MBC) of each essential oil was determined according to the MIC values by transferring 5 µL from MIC tubes to agar plates and incubating at 37 °C for 48 h. The MBC was recorded as the minimum concentration of extract in which no viable bacterial growth was observed. All experimental tests were replicated three different times.

2.6 Statistical analysis

Data were analyzed by one-way analysis of variance with three replications using the SPSS 19.0 statistical software. Means were compared with Duncan test at $p \leq 0.05$ level.

3 RESULTS AND DISCUSSION

3.1 Extraction yield

The color of the ethanol extract from the bulbs of *A. hirtifolium* was light yellow. Statistical analysis indicated that there was significant difference ($p \leq 0.05$) among various populations for extract yield (Table 2). The highest extract yield was obtained from the Samsami population with 14.6% w/w on dry weight basis (Table 2). The lowest value of extract yield was obtained from the bulbs of *A. hirtifolium* collected in Koohrang population with 8.2% w/w on dry weight basis (Table 2). An earlier study by Jafarian et al. (2003) reported the

hydroalcohol extract yield from the bulbs of *A. hirtifolium* collected from Khansar (Isfahan), Iran was 34% using percolation method. In addition, results of a study by Kazemi et al. (2010) indicated the hydroalcohol extract yield from *A. hirtifolium* bulbs was 51.9 g extract obtained from 100 g powder by polyphenolic fraction method. A comparison of our results with the previous reports suggests differences in the extract yield of the plant material could be attributed to extraction methods.

Table 2: Extract yield, antioxidant activity, and total phenolic content of the ethanol extracts from the bulbs of *Allium hirtifolium*

Species	Part used	Populations	Extract yield (% w/w)	Total phenolic (mg GAE /g extract)	IC_{50} (mg/g)
A. hirtifolium	Bulb	Samsami	14.58 ± 3.94 a	38.11 ± 5.06 bc	3.09 ± 0.65 c
A. hirtifolium	Bulb	Khaki	11.12 ± 0.75 b	34.50 ± 4.12 c	2.51 ± 0.61 bc
A. hirtifolium	Bulb	Dasht-e-Laleh	8.17 ± 1.36 bc	44.28 ± 6.58 a	1.90 ± 0.31 b
BHT	-	-	-	-	0.21 ± 0.03 a
ANOVA			$p \leq 0.05$	$p \leq 0.05$	$p \leq 0.01$

†Values in column having similar letter are not statistically different at $p \leq 0.05$

3.2 Total phenolic contents

In present study, total phenolic content in each extract was determined spectrometrically according to the Folin–Ciocalteu method and calculated as gallic acid equivalent (GAE). A significant difference ($p \leq 0.05$) for total phenolic content was measured among the extracts. The maximum total phenolic content was obtained from the extract of the Dasht-e-Laleh population with 44.28 ± 6.58 mg GAE/g extract (Table 2). Results of an earlier study by Ghahremani-majd et al. (2012) indicated that the total phenolic content in the methanol extracts from the bulbs of *A. hirtifolium* populations ranged from 8.4 to 0.5 mg GAE/g sample. Results of a study by Parakesh et al. (2007) indicted that total phenolic contents in the extracts from four (red, violet, white and green) varieties of *Allium cepa* varied from 4.6 to 74.1 mg/g GAE. Within the vegetable family, the composition and quantity of the phenolic are vary significantly according to different intrinsic and extrinsic factors, such as plant genetics and cultivar, soil and growing conditions, maturity state and harvest conditions (Jaffery et al., 2003).

3.3 Antioxidant test

Antioxidant properties are very important in counteracting the deleterious role of free radicals in food and biological systems. In our study, the antioxidant activity of the extract from the various populations of *A. hirtifolium* was expressed as IC_{50} with values from 1.90 to 3.09 mg/ml that indicating the extracts act as moderate to good DPPH scavenger (Table 2). Significant difference ($p < 0.01$) in IC_{50} values were found for the extracts and control (BHT). The extract from the Dasht-e-Laleh population with the highest total phenolic content showed the highest antioxidant activity. Ghahremani-majd et al. (2012) have observed a linear response between total phenolic and antioxidant capacity of the extracts from *A. hirtifolium* bulbs in FRAP, ABTS, and DPPH assays. The antioxidant activity of *Alliums* species was reported by numerous investigators (Velioglu et al., 1998).

3.4 Antibacterial test

The antibacterial activity of the extract from the various populations of *A.hirtifolium* was tested against the four pathogenic bacteria (*Bacillus cereus, Listeria monocytogenes*, *Proteus vulgaris*, and *Salmonella typhimurium*) by using the serial-dilution method. Extracts demonstrated relatively inhibitory activities against the pathogenic bacteria tested, the MICs and MBCs of the tested samples are presented in Table 3. Results of present study indicated that the different bacteria species

demonstrated different levels of sensitivity to the extracts. The MICs of the extracts were within concentration ranges from 0.062 to 0.25 mg/ml, and the respective MBCs were from 0.125 to > 0.50 mg/ml. Generally, the ethanol extracts from the bulbs of *A. hirtifolium* indicated moderate to good inhibitory activities against four bacteria. The highest antibacterial activity was obtained from the extracts of the bulbs of the Dasht-e-Laleh and Samsami populations against *Listeria monocytogenes* and *Bacillus cereus*, respectively. Similarly, results obtained from the measurements of MICs in a study by Ghahremani-majd et al. (2012) showed that *B. subtilis* was the most sensitive microorganism tested to the extracts from the bulbs of *A. hirtifolium* with the lowest MIC values from 1.87 to 15 mg/ml. In addition, they reported the methanol extract from the bulbs of the Isfahan mooseer population had the highest antibacterial and antifungal activities against six bacteria and two fungi. Probably, in present study the phenolic compounds are responsible of the antibacterial activity of the extracts from the bulbs of *A. hirtifolium*. In other study (Amin and Kapadnis, 2005), the extract of bulbs of *A. hirtifolium* had the high antimicrobial activity against wide range of pathogenic and nonpathogenic bacteria and fungi with the MIC values from 0.001 to 0.010 mg/ml .

Table 3: Antibacterial activity (MICs and MBCs) of the ethanol extracts from the bulbs of *Allium hirtifolium* against four bacteria

Species / Antibiotics	Part used	populations	*Bacillus cereus*		*Listeria monocytogenes*		*Proteus vulgaris*		*Salmonella typhimurium*	
			MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
A. hirtifolium	Bulb	Samsami	**62.5**	**125**	125	500	125	500	125	250
A. hirtifolium	Bulb	Khaki	125	250	250	500	125	250	125	250
A. hirtifolium	Bulb	Dasht-e-Laleh	62.5	250	125	250	125	250	125	250
Ciprofloxacin	-	-	**32.2**	**125**	**32.2**	**62.5**	**62.5**	**125**	**62.5**	**125**
Ampicillin	-	-	62.5	125	62.5	125	125	250	125	250

The mechanisms by which plant extracts can inhibit microorganisms vary (Ahmad and Beg, 2001; Rodriguez et al., 2009; Thormar, 2011). Phenolic compounds can act at two different levels: the cell membrane and cell wall of the microorganisms (Taguri et al., 2006). They can interact with the membrane proteins of bacteria by means of hydrogen bonding through their hydroxyl groups which can result in changes in membrane permeability and cause cell destruction (Ghasemi Pirbalouti et al., 2013b).

4 CONCLUSION

The present study is apparently the first report of quantitative total phenol profile, antioxidant and antibacterial activities of the ethanol extracts from the bulbs of *A. hirtifolium* collected from southwestern Iran. The results of current study demonstrated that the ethanol extract from some populations of *A. hirtifolium* with the maximum total phenolic content had the highest antioxidant activity by the DPPH assay. Total phenolic compounds present in the plant are responsible for its effective free radical scavenging, antioxidant and antimicrobial activities. In total, significant antioxidant and antibacterial activities of the extract of the studied herb provide a scientific validation for the traditional use of the plant as an accessible source of natural antioxidants and antimicrobial with consequent health benefits.

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