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Research Article

Preliminar phytochemical screening of the phenolic compounds and antioxidant activity of six *Plantago* species from Iran

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Abstract

pharmaceutical applications. In this study, six different Iranian species of *plantago* were studied to determine their antioxidant activity as well as their total phenolics, flavonoids, flavonols, chlorophyll, carotenoids, and anthocyanin content. The highest contents for total phenolics (92.37 mg GAE/g DW) and total flavonoids (57.16 mg QE/g DW) were found in *P. major*, whereas the highest total flavonols content (46.07 mg QE/g DW) and carotenoids (0.13 mg/g DW) were detected in *P. cornopus*. The least 1,1-diphenyl-2-picrylhydrazy (DPPH) activity (0.66 mg/ml) was found in *P. major*. Also, total antioxidant capacity was in the following order *P. major* > *P. cornopus* > *P. subulata* > *P. lanceolate* ≥ *P. maritima* > *P. ovata*. Preliminar comparison of the Iranian *plantago* species identified them as good sources of phenolic compounds. Hence, *P. major* followed by *P. cornopus* were identified as the richest species in phenolics content with high antioxidant activity.

Key words: Leaf extract, Medicinal, Antioxidant activity, Total phenolics content

Introduction

Medicinal plants, as the "backbone" of traditional medicine, contain natural ingredients that have in recent years been increasingly incorporated in drugs and cosmetic products (Raut and Karuppayil, 2014; Atanasov *et al*., 2015). Plant secondary metabolites play major biological and ecological roles in their resistance and adaptation to environmental stresses (Demain and Fang, 2002). Natural phenolic compounds derived from medicinal plants are used by the food industry as functional ingredients and natural substitutes for synthetic antioxidants (Beara *et al*., 2009; Pollastri and Tattini, 2011). It is conceivable that phenolic compounds (e.g., total phenolics, total flavonoids, and flavonols) might prevent the generation of reactive oxygen species (ROS) in ROS-generating cells. In this context, plants containing sources of bioactive compounds will always be essential for screening new lead compounds (Atanasov *et al*., 2015).

Plantago includes about 256 species, is a genus belonging to the family *plantaginaceae*. These small inconspicuous plants live in many different habitats over the world, including America, Asia, Africa, and Europe (Fons *et al*., 2008). Most *Plantago* plants contain biologically active compounds such as mucilage, flavonoids, sterols, terpenoids, alkaloids, and organic acids (Chiang *et al*., 2002). *Plantago* species have categorized as the most commonly medicinal herbs with a long history of traditional and folk applications (Glen, 1998; Chiang *et al*., 2002; Galvez *et al*., 2005; Fons *et al*., 2008). *Plantago* sp. have been used for its astringent (Fons *et al.*, 2008), anti-toxic (Galvez *et al*., 2005; Adom *et al.*, 2017), antimicrobial (Adom *et al*., 2017), anti-inflammatory (Beara *et al*., 2012), anti-histamine (Adom *et al*., 2017), anti-asthmatic (Galvez *et al*., 2005), diuretic (Fons *et al*., 2008), immune modulating (Fons *et al*., 2008), and wound healing properties (Fons *et al*., 2008) all probably due to their natural antioxidant capacities. In addition, some *Plantago* species have been used in food diets, salads, soups, and herbal tea (Fons *et al*., 2008). So far, twenty-two *Plantago* species have been identified in different geographical regions of Iran, with some ecotypes such as *P. ovata*, *P. evacina*, *P. boissieri*, *P. sharifi*, and *P. major* (Rechinger, 1963).

P. major L., whose main habitats are scattered in Europe and central regions of Asia, is widely distributed in the Hyracanian and Irano-Turanian regions in Iran (Adom *et al*., 2017). Different parts (seed and leaf) of *P. major* have been used for the treatment of a number of diseases, especially chronic bronchitis and infectious diseases while it has also been used for its antibacterial, anti-inflammatory (Adom *et al*., 2017), anti carcinogenic (Beara *et al*., 2009), antivirus (Chiang *et al*., 2002), and wound healing (Adom *et al*., 2017) effects.

P. ovata, commonly known as psyllium, is

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indigenous to Asia (Iran, Pakistan, and India) and the Mediterranean regions of Europe and North Africa (Dhar *et al*., 2011). The leaves and seeds of psyllium are rich sources of phytochemical substances such as mucilage that has been traditionally used to cure high blood pressure, high cholesterol, diabetes, and hemorrhoids (Fons *et al*., 2008; Dhar *et al*., 2011). Also, it has been found that industrial applications are useful as fiber supplement to combat constipation and as thickening, binding, suspending, stabilizing, and gelling agents (Fons *et al*., 2008).

P. lanceolata, as a perennial herb presenting as a wild plant in field crops, is also cultivated for such large consumptions as in pharmaceutical applications (Bajer *et al*., 2016). Different pharmacological properties have been reported for *P. lanceolata* including anti-inflammatory (Beara *et al*., 2012), antimicrobial (Nosrto *et al*., 2000), anti-cancer, and antispasmodic activities (Galvez *et al*., 2005; Beara *et al*., 2012). Herbal tea of *P. lanceolata* is used as folk medicine for healing respiratory disorders (Fons *et al*., 2008).

P. coronopus, is an edible perennial grown as a vegetable plant whose leaves are used as a vegetable in salads (Fons *et al*., 2008). It is native to Eurasia and North Africa (Fons *et al*., 2008) with such known characteristics with high bearing capacity to poor drainage and droughty lands.

P. maritima, native to most of Europe, northwest Africa, and southwest and central Asia, has a sub-cosmopolitan distribution in temperate and coastal regions (Fons *et al*., 2008). The leaves are harvested to be eaten raw and the seeds are also eaten raw or cooked (Fons *et al*., 2008).

P. subulata is found in Anatolia and the Mediterranean Asia. It is acclimated to low-nutrient soils in arid areas but the sea cliffs is its typical habitat (Hassemer, 2018).

Most studies of *Plantago* have been focused on species collected from limited geographical regions. However, given their therapeutic and pharmacological effects, knowledge of *Plantago* sp. species and their applications needs to be urgently documented, especially since several of its species are considered to be neglected crops. *Plantago* species play important roles all over the world because of their vast application due to pharmacological, therapeutic and industrial properties. A number of studies aimed at the phytochemical evaluation of *Plantago* sp. such as *P. altissima* (Beara *et al*., 2012), *P. afra* (Galvez *et al*., 2005), *P. coronopus* (Galvez *et al*., 2005), *P. lagopus* and *P. lanceolata* (Beara *et al*., 2012; Bajer *et al*., 2016) have been reported in the literature. To date, few studies have been conducted on the phenolic compounds or the biochemical content and antioxidant activities of different *Plantago* species, despite their rich phenolic compounds. According to literature review a few study addressing evaluation of different phenolics and antioxidant activity of Iranian *Plantago* species. On the other hand, the existence of a huge gap between phytochemistry and possible ethnopharmacological employments of Iranian *Plantago* species, represents the main aim for this preliminary work. This study provides initial information on these aspects that may eventually lead to a major use of these particular species for different pharmaceutical aims in a next future.

For this purpose, six *plantago* species (namely, *P. ovata*, *P. major*, *P. subulata*, *P. Cornuous*, *P. subulata*, and *P. maritima*) were investigated to identify the variations in their chemical constituents, their bioactive compounds, and antioxidant activities within the framework of three model systems. The study is expected to provide new insight into the more efficient use of these species in food processing and pharmaceutical applications.

Materials and methods

The Iranian accessions from different *Plantago* species (namely, *P. subulata*, *P. cornuous*, *P. maritima*, and *P. lanceolate* and *P. ovata*) were collected from different geographical regions of Iran and identified using the Flora Iranica (Rechinger, 1963) by the experts at the "Pakan Bazr" Botanical Research Institute in Isfahan, Iran. Voucher specimens were deposited at the Herbarium of the Research Institute of Biotechnology and Bioengineering, Isfahan University of Technology, Isfahan, Iran (Table 1).

Leaf extraction procedure: The leaf extracts were prepared according to the methodology reported by Beara *et al*. (2009), with some modifications. Briefly, 200 mg of dried leaf accessions was soaked in 10 ml diethyl ether for 24 hrs. The diethyl ether was concentrated to dryness using a rotary evaporator (Heidolph, Germany) at 45°C until complete evaporation of solvent. Then, 10 ml of aqueous methanol (80:20; v/v, methanol: H_2O) was added for 6 (h). The extracts were filtered (0.45 µm pore size) into clean vials and stored in a refrigerator (4°C), at dark condition for further experiments.

Total phenolics content: Total phenolics content (TPC) was determined according to Beara *et al*. (2009) with minor modifications. A mixture was made combining 0.2 (ml) of folin- ciocalteau's reagent (2N) and 0.5 (ml) of leaf extract. This mixture was kept at room temperature for five minutes. Then, sodium carbonate ($Na_2 CO_3$, 7%, w/v, 2 ml) was added to this mixture, and double distilled water was added until the prepared mixture reach up to 5ml. Another 90 min was required for this mixture to be shaken intermittently in dark. Spectrophotometer (Elico SA 165) was applied to measure the amount of blue color absorbance developed in this way at 725 nm. The same experiment was repeated further two times. The standard curve was constructed by use of gallic acid. The concentration of TPC present in the dry weight (DW) of leaf extract was shown as "mg of GAE/ mg of DW).

Total flavonoids content: Total flavonoids (TFD) content was measured by the method proposed by Akkol *et al*. (2008) with some minor modifications. A

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Table 1. Characteristics of different *Plantago* species used in this study

Accession	Origin	Symbol	Voucher number	Latitude	Longitude	Altitude (m)
P. ovata	Behbahan, Khouzestan, Iran	Po (1)	RIBB/PO-01/2016	30°36' N	50°15' E	325
P. ovata	Saman, Charmahal Bakhtiari, Iran	Po (2)	RIBB/PO-02/2016	31°9' N	50°42' E	1086
P. ovata	Mashhad, Khorasan. Iran	Po (3)	RIBB/PO-03/2016	35°43' N	59°15' E	1050
P. ovata	Khor- Biabanak, Isfahan, Iran	Po (4)	RIBB/PO-04/2016	33°47' N	55°50' E	845
P. ovata	Dehbala, Yazd, Iran	Po (5)	RIBB/PO-05/2016	31°59' N	54°11' E	2600
P. ovata	Tiran, Isfahan , Iran	Po (6)	RIBB/PO-06/2016	32°42' N	51°9' E	1640
P. ovata	Abadeh, Fars, Iran	Po (7)	RIBB/PO-06/2017	31°11' N	52°40' E	2030
P. ovata	Salafchegan, Qom, Iran	Po (8)	-	34°30' N	50°19' E	930
P. major	Tiran, Isfahan , Iran	Pm (1)	RIBB/PM-01/2017	32°42' N	51°9' E	1640
P. major	Birjand, Khorasan, Iran	Pm (2)	RIBB/PM-02/2017	32°53' N	59°13' E	1490
P. lanceolata	Golpayegan, Isfahan, Iran	Pl (1)	RIBB/PL-01/2017	33°45' N	50°27' E	1924
P. lanceolata	Roudsar, Gilan, Iran	Pl (2)	RIBB/PL-02/2017	37°13' N	50°28' E	4
P. subulata	Lahijan, Gilan, Iran	PS	RIBB/PS-01/2017	37°20 N	50°34' E	4
P. cornopus	Kazeroun, Fars, Iran	Pc (1)	RIBB/PC-01/2017	29°62 N	51°65' E	860
P. cornopus	Abadeh, Fars, Iran	Pc (2)	- RIBB/PC-02/2017	31°11' N	52°40' E	2030
P.maritima	Ashkezar, Yazd, Iran	Pma	- *RIBB/PM-01/2017*	31°45' N	54°24' E	1230

mixture was made up of leaf extract (0.5 mL), ethanol 95% (2.5 mL), aluminum chloride 10% (0.2 ml), 1M potassium acetate (0.2 ml) and distilled water (5.6 mL) with a total volume up to 9 ml. This mixture was incubated at room temperature (30 min) and spectrophotometer (Elico SA 165) was applied in order to measure this reaction mixture absorbance at 415 nm. Then, 0.2 ml of distilled water was substituted for 0.2 ml aluminum chloride 10% in blank. Employing a standard curve developed from Quercetin. The content of total flavonoids was stated in terms of quercetin equivalents (QE) as mg per dry weight of leaf (g).

Total flavonols content: A mixture is prepared for total flavonols (TFL) assay containing methanolic extract (1 ml), $AlCl_3$ solution (1 ml, 2% v/v) and sodium acetate (3 ml) (Akkol *et al*., 2008). Then, the absorbance was measured at 445 nm. Total flavonols values were shown as mg quercetin equivalence (QE) in one gram of extract dry weight.

Measurement of photosynthetic pigment: The content of photosynthetic pigments (total chlorophyll, chlorophyll a and b and carotenoid) was estimated by methanolic extract according to Lichtenthaler and Wellburn (1983).

Anthocyanin assay: 100 mg of frozen tissues were soaked in 2 ml of 0.1 NHCl at once. Glass pestle was utilized to crush these tissues. Then, the tissues were kept at room temperature for 3 hrs. A spectrophotometer was used for analyzing absorption at 511 nm of the supernatant of the extract. Finally, Raphanusins coefficient of molar extinction was employed for calculating anthocyanin content (Hara *et al*., 2003).

DPPH assay: According to Salem *et al*. (2011), after minor modifications, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) was employed for determining the radical activities of *Plantago sp*. for scavenging. 1 mL of DPPH solution in methanol (50μM) was mixed with 20 μl of the extract. The extracts of the leaf were examined in concentrations of 200, 400, 900 and 1800 μg/ml .The mixtures produced with each of these concentrations were incubated in the dark for 20 min after mixing well. DPPH absorption reduction was measured at 515 nm. As a positive control, ascorbic acid (AA) was chosen to be employed. All the required measures were determined in triplicate. The following equation was utilized in order to calculate the radical scavenging activity of DPPH: The inhibition percentage (IP %) was estimated as:

$$IP\ (\%) = [A_{control} - A_{sample} / A_{control}] \times 100$$

Where the control was regarded as the control reaction absorbance and the sample was the absorbance when plant extract was present. The regression equation containing extract concentrations and inhibition concentration (IC) was used for calculating extract concentration providing 50% inhibition.

Phosphomolybdate assay: Phosphomolybdate (PM) assay determined the leaf extract total antioxidant capacity with ascorbic acid as a standard (Saeed *et al*., 2012). For this assay, 0.1 ml of the extract was mixed with 3 ml of reagent solution. The reagent solution was composed of sulfuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM). The reaction solution present in the tubes underwent incubation at 95°C for an hour and half. Then, the samples were cooled at room temperature. Subsequently, the solution absorbance was calculated at 695 nm against blank. Methanol (0.1 ml) was replaced for the extract as the blank. Standard graph of ascorbic acid was employed in order to calculate the equivalents of ascorbic acid.

β- carotene -linoleic assay: Antioxidant activity was assessed using the β- carotene - linoleate model system with slight modification according to Kulisic *et al*. (2004). To begin, 2 mg of β- carotene was dissolved in chloroform (1.0 ml). Then, a mixture was made from combining linoleic acid (Sigma, 200 μl) and tween-80 (0.2 ml). This mixture was allowed to stay at room temperature for 15 min. Subsequently, oxygenated distilled water (50 ml) was added to this mixture after chloroform evaporation, and the mixture was shaken

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until linoleic- β - carotene emulsion was produced. Test tubes containing different concentrations (200, 400, 900 and 1800 µg) of the extract (0.2 ml from each) were employed to test this emulsion. The linoleic- β-carotene emulsion (3.0 ml) was transferred into these tubes, then shaken and incubated in a water bath at a temperature of 50ºC. The zero time absorbance (A_0) at 470 nm and A_1 (after passing120 min) was measured by a spectrophotometer. As the background subtraction, a blank without β- carotene, was employed. The following equation was utilized to calculate lipid peroxidation inhibition:

LPO inhibition (%) = 1- $A_1/A_0 \times 100$

The standard was set to be butylated hydroxytoluene (BHT) (R^2= 0.96).

The analysis of variance was carried out using PROC ANOVA of SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA 2011) using a completely randomized design with three replicates. Then, mean comparisons were carried out using Fisher's least significant difference ($LSD_{5\%}$) test to determine differences among and within species. The results were expressed as means ± S.E (standard error). The correlation coefficients between the bioactive components were calculated using the proc CORR of SAS.

Results and discussion

The results of analysis of variance showed the significant effects of entries and entries (genotypes) on all the studied traits (Table 2).

Total phenolics content: Total phenolics content serves as an antioxidants which counteracting with reactive oxygen species (ROS) under oxidative stresses (Singleton *et al*., 1999; Quideau *et al*., 2011). Phenolic compounds contribute to the transfer of hydrogen to free radicals to produce a stabilized form of phenoxide radical (Jankovic *et al*., 2012). The capacity of phenolics to scavenge free radicals may be due to the many phenolic hydroxyl groups they possess (Jankovic *et al*., 2012). In this study, the TPC of 16 accessions varied in the range of 105.9 (mg of GAE/g DW) in *P. major* (2) to 31.01 (mg of GAE/g DW) in *P. ovata* (1) (Table 3). Beara *et al*. (2009) reported that TPC ranged from 38.43 to 70.97 (mg of GAE/g of DW) in different *Plantago* species including *P. argentea, P. holosteum, P. major* L., *P. maritima* L., and *P. media* L. The amounts of TPC in leaf extracts from the different Iranian species followed the following order: *P. major* (92.73 mg GAE/g DW) > *P. cornopus* (86.83 mg GAE/g DW) > *P. lanceolata* (76.51 mg GAE/g DW) > *P. maritima* (60.14 mg GAE/g DW) > *P. subulata* (55.21 mg GAE/g DW) > *P. ovata* (50.04 mg GAE/g DW) (Table 3). Varying amounts of TPC have been reported in *P. lanceolata* (8.16-24.97 mg GAE/g DW) (Galvez *et al*., 2005; Jankovic *et al*., 2012) and *P. cornopus* (7.11-9.25 mg GAE/g DW) (Galvez *et al*., 2005; Jankovic *et al*., 2012). Based on our findings, *P. major* and *P. cornopus* are rich sources of TPC, which is in agreement with those reported elsewhere (Beara *et al*., 2009; Pereira *et al*., 2017). Differences in geographical region (Zhang *et al*., 2015), variety, species (Quideau *et al*., 2011), plant organ, plant growth stage (Pierie *et al*., 2017), plant ecotype, solvent (Koleva *et al*., 2002; Gharibi *et al*., 2013; Do *et al*., 2014), and the extraction method used (Koleva *et al*., 2002; Quideau *et al*., 2011; Do *et al*., 2014) have been claimed to have marked effects on the quantity and types of phenolics content.

Total flavonoids content: Flavonoids are key components of the antioxidant system (Magney, 2017). Belonging to the polyphenolic compounds, they have a diverse range of functions in the plant defense system like serving as chemical messengers or free radical scavengers (Akkol *et al*., 2008; Magney, 2017). Flavonols and anthocyanins are considered as two major subgroups of flavonoids (Magney *et al*., 2017) that are considered as one of the most frequent secondary metabolites named flavon chemotypes (Beara *et al*., 2009) in such *Plantago* species as *P. lanceolata* and *P. altissima*. Among the different accessions, TFD showed a broad variations from 30.13 (mg QE/g DW) in *P. ovata* (2) to 66.12 (mg of QE/g DW) in *P. major* (2) (Table 3). Varying values for TFD have been reported in other *Plantago* species including *P. major* (5.31 mg of QE/g of DW) (Beara *et al*., 2009), *P. maritima* (12.93 mg of QE/g DW) (Beara *et al*., 2009), *P. cornopus* (146 mg of RE/g) (Piera *et al*., 2017), *P. argentea* (6.77 mg of QE/g of DW) (Beara *et al*., 2009), and *P. holosteum* (13.10 mg of QE/g of DW) (Beara *et al*., 2009). Differences in total flavonols content within and between the species may be attributed to differences in extraction methods, analytical methods, and inter-cultivar variability (Mageney *et al*., 2017). For example, Piera (2017) found that their methanolic extract yielded a higher total flavonoids content from *P. cornopus* than did ethyl acetate or hexane solvents. Regarding the content of total flavonoids among different species, the highest content was measured in *P. major* (57.16 QE/g DW) and the lowest in *P. subulata* (34.54 QE/g DW) (Table 3). Moreover, *P. major* differed significantly in TFD quantity from other *Plantag*o species (Table 3), a finding that was different from that reported by Beara *et a*l. (2009). It may be concluded that the TFD content of *P. major* should be linked to its anticancer (Beara *et al*., 2009) and anti-inflammatory (Adom *et al*., 2017) effects.

Total flavonols content: Flavonols are present in a wide variety of species and are the most important factor in plant response to stresses as represented by their antioxidant activity (Pollastri and Tattini, 2011). Our literature review showed no reports on the quantities of total flavonols in *Plantago* SP. As shown in Table 3, TFL content varied from 51.40 mg of QE/g DW in *P. cornopus*-2 to 15.87 in *P. ovata*-7. Given the broad range of physiological effects of flavonols such as protection against sunlight damage, herbivores, and microbes (Pollastri and Tattini, 2011), superior species such as *P. cornopus* could be used as nutraceuticals in

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Table 2. Analysis of variance for different biochemical studied traits in different *Plantago* species.

S.O.V		TPC¥	TFD	TFL	ANT	Chla	Chlb	ChlT	Car	DPPH	PM	B-Car
Entries												
Species	5	1740.85**	307.07**	436.95**	2.23**	0.26**	0.17**	0.76**	0.0025**	0.157**	0.27**	1.81**
Species (genotype)	10	409.6**	106.4	44.16	0.58**	0.20**	0.03	0.45**	0.0034**	0.23**	0.09**	1.07**
Residual	16	14.48	66.41	18.83	0.0018	0.0005	0.019	0.0011	0.0003	0.002	0.0026	0.0007

***, ** Significant at 5% and 1% of probability. ¥ TFC: total phenolics content; TFD: total flavonoids; TFL: total flavonols; Ant: anthocyanin, Chla: Chlorophyll a; Chlb: Chlorophyll b; ChlT: Chlorophyll total; Car: Carotenoid; DPPH: 2,2-diphenyl-1-picrylhydrazyl; PM: phosphomolibdate assay; β-car: β carotene- linoleic acid assay.**

Table 3. Evaluation of total phenolics, total flavonoids, total flavonols, anthocyanin, chlorophyll, and carotenoid contents in the 16 *plantago* accessions.

Accession	TPC¥	TFD	TFL	Ant	Chla	Chlb	ChlT	Carotenoids
	(mg GAE/g DW)¥	(mg QE/g DW)	(mg QE/g DW)	(µmol/ g FW)	(mg/g DW)	(mg/g DW)	(mg/g DW)	(mg/g DW)
Po(1)Ɨ	31.01^{k}±1.41	30.26^{C}±0.60	20.56^{cd}±5.74	1.44^{i}±0.02	0.42^{i}±0.01	0.28^{de}±0.02	0.73^{g}±0.04	0.07^{c}±0.002
Po(2)	33.94^{jk}±5.73	30.13^{c}±1.38	21.65^{cd}±2.43	2.09g±0.04	0.52^{h}±0.03	0.4^{cde}±0.02	0.91^{f}±0.02	0.09^{bc}±0.002
Po(3)	40.87^{ij}±4.44	34.55^{bc}±10.52	20.50^{cd}±5.83	1.9^{h}±0.02	0.57^{gh}±0.57	0.38^{cde}±0.02	0.93^{f}±0.01	0.08^{bc}±0.004
Po(4)	45.49^{i}±4.67	32.3^{c}±10.60	22.18^{cd}±6.18	1.86^{h}±0.02	0.52^{h}±0.52	0.43^{b-e}±0.02	0.98^{f}±0.04	0.12^{ab}±0.013
Po(5)	66.84ef±3	53.14^{ab}±12.14	26.12^{cd}±0.40	2.24^{f}±0.04	0.81^{e}±0.01	0.47^{b-e}±0.02	1.26^{d}±0.01	0.05^{c-f}±0.006
Po(6)	56.72^{gh}±2.88	36.44^{bc}±10.70	20.46^{cd}±5.43	1.49^{i}±0.01	0.40^{i}±0.01	0.27^{de}±0.02	0.65^{h}±0.02	0.04^{c-f}±0.001
Po(7)	64.25^{fg}±2.3	38.08^{bc}±12.81	15.87^{d}±0.97	0.96^{j}±0.02	0.33^{j}±0.02	0.16^{e}±0.02	0.48^{i}±0.01	0.08^{bc}±0.001
Po(8)	61.24^{fgh}±3.4	40.19^{bc}±6.81	25.62^{cd}±0.88	2.32^{f}±0.07	0.61^{fg}±0.61	0.53^{bcd}±0.02	1.16^{e}±0.01	0.05^{def}±0.001
Pm(1)	78.81^{cd}±3.06	48.21^{abc}±1.64	26.93C±5.4	1.84^{h}±0.08	0.28^{jk}±0.01	0.51^{bcd}±0.05	0.45^{i}±0.04	0.01^{f}±0.001
Pm(2)	105.9^{a}±1.24	66.12^{a}±4.01	40.40^{b}±0.21	3.04^{c}±0.02	1.24^{b}±1.24	0.64^{abc}±0.006	1.86^{b}±0.006	0.08^{cde}±0.002
Pl(1)	78.67^{cd}±6.56	36.53^{bc}±5.23	27.96^{c}±4.02	3.70^{a}±0.01	1.08^{c}±0.02	0.75^{ab}±0.02	1.91^{b}±0.04	0.01^{ef}±0.008
Pl(2)	74.36^{de}±7.69	34.67^{bc}±5.10	28.12^{c}±0.35	3.40^{b}±0.06	0.90^{d}±0.03	0.52^{bcd}±0.02	1.08^{e}±0.04	0.06^{cd}±0.001
PS	55.21^{h}±1.55	34.54^{bc}±9.92	21.09^{cd}±1.94	2.72^{d}±0.03	0.32^{j}±0.03	0.53^{bcd}±0.02	1.43^{c}±0.032	0.01^{ef}±0.004
Pc(1)	90.36^{b}±0.93	40.81^{bc}±9.92	40.75b±0.52	2.56^{e}±0.02	1.42^{a}±0.03	0.87^{a}±0.02	2.34^{a}±0.06	0.12^{a}±0.002
Pc (2)	83.31^{bc}±0.49	42.95^{bc}±1.97	51.40a±2.87	3.12^{c}±0.04	0.86^{d}±0.01	0.9^{a}±0.02	1.41^{c}±0.04	0.14^{a}±0.003
Pm	60.15^{fgh}±0.46	39.44^{bc}±9.54	24.25^{cd}±5.74	2.09^{g}±0.04	0.62^{f}±0.01	0.47^{de}±0.02	1.10^{e}±0.02	0.08^{bc}±0.004
Species								
P. ovata	50.04^{e}±13.84	36.76^{b}±9.70	21.62^{c}±4.89	1.79^{f}±0.44	0.52^{e}±0.14	0.35^{c}±0.11	0.89^{e}±0.25	0.07^{b}±0.023
P. major	92.37^{a}±15.76	57.16^{a}±10.63	33.67^{b}±8.36	2.44^{d}±0.7	0.76^{c}±0.52	0.57^{abc}±0.32	1.15^{d}±0.81	0.06^{bc}±0.06
P. lanceolata	76.51^{b}±14.09	35.60^{b}±4.35	28.04^{bc}±2.33	3.55^{a}±0.66	0.99^{b}±0.43	0.7^{ab}±0.21	1.5^{b}±0.47	0.08^{b}±0.076
P. subulata	55.21 ^{d} ±7.69	34.54^{b}±9.92	21.09^{c}±1.2	2.64^{c}±0.03	0.89^{c}±0.03	0.53^{bc}±0.03	1.43^{c}±0.03	0.038^{c}±0.009
P. cornopus	86.83^{a}±4.11	41.88^{b}±5.97	46.07^{a}±8.37	3.26^{b}±0.17	1.44a±0.32	0.81^{a}±0.07	1.87^{a}±0.54	0.13^{a}±0.004
P. maritima	60.14^{c}±0.46	39.44^{bc}±9.54	24.25^{c}±5.74	2.09^{e}±0.04	0.62^{d}±0.02	0.53^{bc}±0.03	1.1^{d}±0.02	0.08^{b}±0.003

¥TFC: total phenolics content; TFD: total flavonoids; TFL: total flavonols; Chla: Chlorophylla; Chlb: chlorophyllb; ChlT: Chlorophyll total; Ant: Anthocyanin. Means (± standard errors) with different letters in each column are statistically significant at 5% level probability. Ɨ: The abbreviations are the same as those used in Table 1.

food products or as species of special interest in medical researches. Comparison of means among the *Plantago* species with respect to TFL showed a significant difference between *P. major* and *P. cornopus* (P <0.05), but none among other species (Table 3). The highest content for TFL (46.07 mg QE/g DW) belonged to *P. cornopus* among the species investigated whereas the least (21.09 mg QE/g DW) belonged to *P. subulata* (Table 3).

Chlorophyll (a, b) content: Plant photosynthetic capacity is directly determined by its chlorophyll content (Gitelson *et al*., 2009; Hughes *et al*., 2011). Among the studied accessions, the highest values of ChlT (2.34 mg/g DW) and Chla (1.42 mg/g DW) were observed with *P. cornopus* -1 but the least values of ChlT (0.45 mg/g DW) and Chla (0.28 mg/g DW) belonged to *P. major*-1. For Chlb, both genotypes of *P. cornopus* showed the highest mean, but its least value belonged to those of *P. ovata* (Table 3). On the other hand, different genotypes of *P. ovata* showed no significant differences in their Chlb content (Table 3). Different chlorophyll parameters could be considered as an index of interspecific differences and the adaptability of different species to luminosity (Aurelia, 2013). Mean

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comparisons among the evaluated species showed that *P. cornopus* recorded the highest values for ChlT (1.87 mg/g DW), Chla (1.44 mg/g DW), and Chlb (0.81 mg/g DW) whereas the least values of ChlT (0.89 mg/g DW), Chla (0.52 mg/g DW), and Chlb (0.36 mg/g DW) belonged to *P. ovata* (Table 3). In contrast to these findings, Aurelia (2013) reported the following order for Chla: *P. lanceolate* > *P. maritima* > *P. cornopus* with no significant differences in Chlb content. Finally, it has been observed that leaf pigments (chlorophyll and carotenoid) as indicators of plant vigor and photosynthetic capacity in *Plantago* depend on species, genotype, as well as ontogenetic moments (Aurelia, 2013).

Carotenoids content: Carotenoids, as a class of phyto-nutrient pigments, play a major role in the protection of plant cells against reactive oxygen species (Xu *et al*., 2017). Plant species with high carotenoid contents have attracted attention for curing inflammatory and cancer disorders (Xu *et al*., 2017). Among the accessions evaluated, the highest carotenoid content belonged to *P. cornopus* genotypes, whereas the least (0.01 mg/g DW) belonged to *P. major* (1). Among the species evaluated, the highest carotenoid content (0.13 mg/g DW) was measured in *P. cornopus*, whereas the least (0.038 mg/g DW) belonged to *P. subulata* (Table 3). Considering the role of carotenoids as non-enzymatic antioxidants, *P. cornopus* leaf may be used as a rich source of natural antioxidant for scavenging free radicals.

Anthocyanin content: Anthocyanin, as a subgroup of flavonoids, is characterized by antiviral, antibacterial, anti-cancer, and anti-inflammatory activities as well as improving effects on visual acuity (He *et al*., 2010; Xu *et al*., 2017). Anthocyanin content ranged from 0.96 (µmol/g FW) in *P. ovata* (7) to 3.70 (µmol/ g FW) in *P. lanceolata* among the different accessions investigated (Table 3). Averaged over all species, *P. lanceolata* (3.55 µmol/g FW) and *P. ovata* (1.79 µmol/g FW) were found to be the richest and weakest sources of anthocyanin, respectively. The present study reports for the first time on the quantification of anthocyanin content in different *Plantago* species. Based on our results and given its high anthocyanin content, *P. lanceolata* may be recommended as a natural source of non-enzymatic antioxidant and free radical scavenger for use by the food industry (Xu *et al.*, 2017).

Antioxidant assay: DPPH free radical scavenging is generally accepted as a main mechanism against lipid oxidation (Koleva *et al*., 2002). The effect of antioxidants on DPPH radical scavenging has been thought to be due to their hydrogen donating ability. Among the different accessions studied, the highest (1.80 mg/mL) and lowest (0.52 mg/mL) DPPH activities were observed in *P. ovata* (8) and *P. major* (2), respectively (Table 4). Data revealed that *P. ovata* (8) had a higher antioxidant activity in quenching DPPH free radicals than did other extracts. The differences in IC_{50} observed between the samples could be described by variations in polyphenolic composition of the extracts analyzed (Gharibi *et al*., 2ح013). Similar to these findings, Beara *et al*. (2009) reported a lower DPPH activity in *P. major* (5.35 µg/mL) than in *P. maritima* (6.79 µg/mL), indicating the higher antioxidant capacity of *P. major* than that of *P. maritima*. The inferior IC_{50} (4.20 µg/mL) of *P. lanceolata* to that of *P. altissima* (IC_{50}=10.74 µg/mL) reported by Beara *et al*. (2012) is in agreement with the high antioxidant activity of *P. lanceolata*. Contradictory results have, however, been reported on the DPPH activity of *P. cornopus* as evidenced by the IC_{50} value of 1.21 mg/mL reported by Pereira *et al*. (2017), which is similar to our finding, and that of 47.43µg/mL reported by Galvez *et al*. (2005), which is different from the value measured in the present study.

This study reports for the first time on the antioxidant activity of *P. ovata* evaluated by the DPPH assay. This antioxidant evaluation method had been previously employed for *P. lanceolata* (Beara *et al*., 2012; Galvez *et al*., 2005), *P. major* (Beara *et al*., 2009; Selamoglu *et al*., 2017), *P. cornopus* (Galvez *et al*., 2005; Pereira *et al*., 2017), *P. maritima* (Beara *et al*., 2009), and such other *Plantago* species as *P. serraria* (7.60 µg/ml) and *P. lagopus* (31.31 µg/ml) (Galvez *et al*., 2005). DPPH activity in the species evaluated was observed to follow the order: *P. maritima* (1.52 mg/ml > *P. lanceolata* ≥ *P. ovata* (1.22 mg/ml) > *P. cornopus* (1.1 mg/ml) > *P. subulata* (0.8 mg/ml) > *P.major* (0.66 mg/mL) (Table 3). Moreover, the mean values of DPPH activity in the evaluated species showed that the extracts from *P. major* and *P. subulata* exhibited higher antioxidant activities (the lowest IC_{50} values) than did ascorbic acid (1 mg/mL) used as the positive control, while the extract from *P. maritima* showed the lowest activity (Table 3). The high antioxidant activity of the superior species examined in this study may be attributed to the high variations in their polyphenolic compositions (Beara *et al*., 2012; Gharibi *et al*., 2013) or the effects of low temperature at high altitudes that lead to the greater biosynthesis of some kinds of antioxidants such as ascorbic acid (Gharibi *et al*., 2013).

A free radical-mediated method involves beta-carotene bleaching resulting from the hydroperoxides formed by air oxidation of linoleic acid (Kulisic *et al*., 2004). The method has been employed for the evaluation of antioxidant activity in different medicinal plants such as *oregano* (Kulisic *et al*., 2004), safflower (Salem *et al*., 2011), Achillea (Gharibi *et al*., 2013), and some species of *Plantago* such as *P. lanceolata* and *P. major* (Selamoglu *et al*., 2017). The highest IC_{50} (4.48 mg/ml) belonged in *P. maritima* (2) while the lowest (IC_{50}= 0.37 mg/ml) was denoted to *P. ovata* (8). These results imply that different accessions within a *Plantago* species might exhibit significant differences in their antioxidant systems examined via enzymatic assays (Table 4).

The β-carotene bleaching used to evaluate the antioxidant activities of six *Plantago* species ranked

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Table 4. Evaluation of different antioxidative assay models (DPPH, β-car and PM assay) in different *Plantago* accessions

Accession	DPPH[¥](mg/ml)	β-car (mg/ml)	PM (mg AAE/g FW)
Po(1) [Ɨ]	1.04^{fgh} ± 0.04	1.67^{h} ± 0.02	0.71^{fg} ± 0.02
Po(2)	1.01^{gh} ± 0.02	1.40^{j} ± 0.01	0.70^{g} ± 0.007
Po(3)	1.24^{d} ± 0.01	1.60i ± 0.02	0.73^{efg} ± 0.01
Po(4)	1.12^{ef} ± 0.02	1.99^{e} ± 0.01	0.85^{d} ± 0.007
Po(5)	0.86^{i} ± 0.02	1.91f ± 0.02	1.13^{b} ± 0.01
Po(6)	1.17^{de} ± 0.07	1.44^{j} ± 0.017	0.84^{de} ± 0.05
Po(7)	1.50^{b} ± 0.03	1.22^{k} ± 0.004	0.36^{h} ± 0.02
Po(8)	1.80^{a} ± 0.06	0.37^{hi} ± 0.02	0.89^{cd} ± 0.01
Pm(1)	0. 8^{i} ± 0.04	1.74^{g} ± 0.01	1.56^{a} ± 0.01
Pm(2)	0.52^{j} ± 0.02	1.12^{l} ± 0.02	1.58^{a} ± 0.02
Pl(1)	0.97^{h} ± 0.03	2.0^{d} ± 0.05	1.21^{b} ± 0.01
Pl(2)	1.50^{b} ± 0.03	2.19^{c} ± 0.02	0.71^{fg} ± 0.03
PS	0.80^{i} ± 0.06	1.73^{g} ± 0.008	1.08^{b} ± 0.09
Pc (1)	1.08^{efg} ± 0.05	1.72^{g} ± 0.018	0.98^{c} ± 0.05
Pc (2)	1.12^{ef} ± 0.021	3.23^{b} ± 0.04	1.24^{b} ± 0.09
Pm	1.52^{b} ± 0.076	4.48^{a} ± 0.04	0.9c ± 0.11
Synthetic antioxidant (control)	1.01 ± 0.03	1.51 ± 0.08	
Species			
P. ovata	1.22^{b} ± 0.29	1.61^{e} ± 0.24	0.77^{d} ± 0.20
P. major	0.66^{e} ± 0.49	1.43^{f} ± 0.01	1.56^{a} ± 0.01
P. lanceolata	1.23^{b} ± 0.03	2.13^{c} ± 0.07	0.96^{c} ± 0.30
P. subulata	0.8^{d} ± 0.06	1.73^{d} ± 0.01	1.08^{b} ± 0.09
P. cornopus	1.1^{c} ± 0.04	2.48^{b} ± 0.86	1.11^{b} ± 0.01
P. maritima	1.52^{a} ± 0.07	4.48^{a} ± 0.04	0.9^{c} ± 0.11

[¥]:DPPH: 2,2-diphenyl-1-picrylhydrazyl; PM: phosphomolibdate assay; β-car: β carotene-linoleic acid assay.
Means (± standard errors) with different letters in each column are statistically significant at 5% level probability. Ɨ: The abbreviations are the same as those used in Table 1.

them in the following ascending order: *P.maritima* (4.48 mg/ml) < *P.cornopus* (2.48 mg/ml) < *P. lanceolata* (2.13 mg/ml) < *P.subulata* (1.73 mg/ml) < *P.ovata* (1.61 mg/ml) < BHT as synthetic antioxidant (1.51 mg/ml) < *P. major* (1.43 mg/ml) (Table 4). In agreement with this finding, Selamoglu *et al.* (2017) reported that β-carotene assay revealed a higher antioxidant activity in *P. major* than in *P. lancelata*.

Total antioxidant capacity (TAC) was expressed in ascorbic acid equivalent through PM assay. The method was previously in such other medicinal plants as *Torilis leptophylla* (Saeed *et al*., 2012); there has been, however, no report in the literature indicating the application of this method to *Plantago* species. The antioxidant capacities of various *Plantago* species varied from 1.58 (mg AAE/g FW) in *P. major*-2 to 0.36 (mg AAE/g FW) in *P. ovata* -7 (Table 4). The TAC was more pronounced in *P. major* than in the other species evaluated (Table 4). Differences in specificity and sensitivity of each evaluation method used make it impossible to use equal amounts of antioxidants for each test (Koleva *et al*., 2002; Kulisic *et al.*, 2004). The different antioxidant activities of different accessions as determined by enzymatic assays suggested that different accessions employed different mechanisms such as prevention of radical chain initiation, decomposition of peroxides, or prevention of hydrogen abstraction (Koleva *et al*., 2002). This justifies the application of multi-concentration methods to provide a more comprehensive perspective of the antioxidative activity in *Plantago* species.

Correlation analysis: Correlation coefficients among different chemical constituents were calculated using three antioxidant activity model systems (Table 5). Total pgenolics content exhibited positive correlations with TFD (r=0.75**) and TFL (r=0.76**) (Table 5). Similar results had been reported for such other species as *Limnophila aromatica* (Do *et al*., 2011), *Buxifolia* (Jan *et al*., 2013), and *Torilis leptophylla* L. (Saeed *et al*., 2012). The positive correlation between TPC and TFL as well as that between TPC and TFD could be attributed to their similar biosynthetic pathways (Hara *et al.*, 2003) in different *Plantago* species. Since flavonoids make up the largest group of phenolic compounds, the two flavonoid subgroups (flavonols and anthocyanins) were both positively associated with high antioxidant capacity (PM assay) (Table 5). Our correlation analysis revealed that the results of enzymatic antioxidant assays agreed well with antioxidant activities observed in natural environments. Significant correlations were established among TPC, TFL, and TFD by PM assays (Table 5) in agreement with those reported by Jan *et al*. (2013) for *Monotheca Buxifolia*. A negative correlation was, however, established between TFL and DPPH (r = – 0.49*), demonstrating the effective antioxidant activity of *Plantago* flavonols in scavenging free radicals.

The antioxidant activities of the extracts in this study could be attributed to the polyphenolic compounds neutralizing the linoleate free radical and other free radicals formed in this model system, which oxidize unsaturated β-carotene molecules (Kulisic *et al*., 2004) No significant correlation was found between TPC and β-car assays (Table 5). Considering the fact that β-car

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Table 5. Correlation between the different traits studied including TPC, TFD, TFL, Chla, Chlb, ChlT, Ant, and the antioxidant assays in different *Plantago* species.

	TPC	TFD	TFL	Ant	Chla	Chlb	ChlT	Car	DPPH	PM	β-car
TPC	1										
TFD	0.75**	1									
TFL	0.76**	0.79**	1								
Ant	0.57*	0.24	0.52*	1							
Chla	0.7**	0.44	0.48	0.69**	1						
Chlb	0.67**	0.33	0.60*	0.80**	0.75**	1					
ChlT	0.59*	0.33	0.36	0.73**	0.88*	0.82**	1				
Carotenoids	0.005	-0.05	0.034	-0.09	0.31	0.19	0.17	1			
DPPH	-0.27	-0.46	-0.49*	-0.21	-0.19	-0.25	-0.29	0.18	1		
PM	0.65**	0.72**	0.85**	0.50*	0.36	0.61*	0.42	-0.27	-0.67**	1	
β-car	0.1	-0.01*	0.13	0.22	0.11	0.27	0.10	0.29	0.05	0.13	1

¥TPC: total phenolics content; TFD: total flavonoids; TFL: total flavonols; Ant: anthocyanin; Chla: chlorophyll a; Chlb: Chlorophyll b; ChlT: chlorophyll total; Car: Carotenoids; DPPH: 2,2-diphenyl-1-picrylhydrazyl; PM: phosphomolibdate assay; β-car: β carotene-linoleic acid assay.

assay employs an emulsified lipid and is especially applicable for investigating lipophilic antioxidants (such as the antioxidant activity of essential oils), it could be concluded that the lipophilic antioxidants detected in the evaluated species exhibit no high potential for scavenging free radicals. Significant and positive correlations were established between Ant content, on one hand, and ChlT ($r = 0.73^{**}$), Chla ($r = 0.69^{**}$), Chlb ($r = 0.80^{**}$), and DPPH activity ($r = 0.5^{*}$), on the other (Table 4). However, it should be noted that Ant in *Plantago* play equal roles in both photo protection (Hughes *et al.*, 2011) and antioxidant activities.

Conclusion

Much research has been devoted in recent years to the utilization of medicinal and aromatic plants in cosmetic, food, and pharmaceutical products. Medicinal plants characterized by a strong antioxidant activity might be used as a safe source in food products. The phytochemical evaluation of Iranian species of *Plantago* showed that *P. ovata*, *P. cornopus* and *P. subulata* contained useful effects because of their phenolics extracts. *P. major* and *P. cornopus* recorded the highest quantities of phenolic compounds with high antioxidant activities. In addition to the high antioxidant activity of leaf extracts, along with other phenolics in leaf can be beneficially used by the food industry despite their low quantities as they offer pharmaceutical benefits to fill the gaps and to remedy the inadequacies in current therapeutic options. Clearly, selection of more accessions in each species might be useful to achieve more insightful results.

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