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Total Phenolic Content and Antioxidant Activity of *Fumaria vaillantii* Extract at Three Phenological Stages Assayed by Various Methods

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Abstract
Fumaria vaillantii (Fumariaceae) is an annual plant which is growing in a wide diversity area of Iran which has been used traditionally in the folk medicine. Antioxidants have great importance to reduce oxidative stress and prevention of damage to biological molecules. The present study was designed to investigate the antioxidant activity of ethanolic extract from aerial parts of *Fumaria vaillantii* L. by using three different methods including 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Ferric reducing antioxidant power (FRAP) and Phosphomolybdenum complex at three different growth stages. In addition, total phenolic contents of the extracts were determined using Folin-Ciocalteu method. According to the obtained results, the total phenol contents of aerial parts harvested at vegetative, budding and flowering stages were 68.38, 71.11 and 56.42 mg GAE/g extract, respectively. Antioxidant activities of the extracts were decreased from vegetative to flowering stages in DPPH and FRAP methods. In conclusion, ethanolic extract from vegetative stage of Iranian *F. vaillantii* can be considered as a potential source of natural antioxidants for food and pharmaceutical industries.

Keywords: Fumitory, DPPH-radical, Folin-Ciocalteu method, growth stage, medicinal plant

Abbreviations: ***F. vaillantii****, Fumaria vaillantii* L.; **DPPH**, 2, 2-diphenyl-1-picrylhydrazyl; **FRAP,** Ferric reducing antioxidant power; **BHA,** butylated hydroxy anisole; **BHT**, butylated hydroxy toluene; **TBHQ**, tertiary butylhydro quinine.

Introduction

The genus *Fumaria* L. (Fumariaceae) is comprised of 60 species, which most of them grow around the Mediterranean region. In English, *Fumaria* species is known as "fumitory, earth smoke, beggary, fumus, vapor, fumittery or wax dolls". The best way to distinguish *Fumaria* species, is studying some characteristics such as sepal size, upper petal shape and dry fruit shape (Murphy, 2009). In total, eight species from this genus have been reported from Iran (Ebrahimzadeh Araii et al., 2011). *Fumaria vaillantii* is an annual plant which is growing in a wide area of Iran. It is a small herb with numerous rootlets and root hairs, light green smooth stem, 2-4 mm in diameter, long linear leaves divided into narrow segments, and pink flowers in racemes (Rajopadhye and Upadhye, 2011). It is mainly grown in the cooler region of the world (Ebrahimzadeh Araii et al., 2011). In Iran, it is distribute in

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Mazandaran, eastern and western of Azarbayjan, Krdestan, Kermanshah, Hamedan, Esfahan, Yazd, Kerman, Khorasan, Semnan and Tehran provinces (Mozafarian, 2012). It has been used traditionally in the folk medicine for treatment of hepatobiliary, dysfunction and gastrointestinal disorders, dermatological diseases and as a blood purifier (Ebrahimzadeh Araii et al., 2011). Moreover, *Fumaria* species contain different types of fatty acids, such as phospholipids, with antioxidant effects (Gupta et al., 2012).

Fumaria plants have various phytochemicals such as phenolic diterpenes, flavonoids and phenolic acids which are potential sources of natural antioxidants. So they have been shown to have anticancer and anti-carcinogenic activities (Cesoniene et al., 2012). Plant extracts and their constituents are known to exert biological effects, especially antioxidant activity (Gursoy and Tepe, 2009). Therefore, the use of these plants based on their natural antioxidants for different purposes such as food industries, preventive materials and therapeutic medicines have been recently drawn interests (Chen et al., 2008).

Antioxidants are able to protect the human body from oxidative damage associated with the reaction of free radicals. They act as radical scavengers; inhibit lipid peroxidation and other free radical- mediated processes. Synthetic antioxidants such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and tertiary butylhydro quinone (TBHQ) are known to have toxic and carcinogenic effects on human health, therefore their application have been limited (Sultana et al., 2007; Cesoniene et al., 2012). In this study the antioxidant activity of *F. vaillantii* extracts at three growth stages of *F. vaillantii* were evaluated. The ethanol extracts were screened for their antioxidant activity using three methods as 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging test, ferric-ion reducing antioxidant power (FRAP) and phosphomolybdenum complex. Also, total phenolic was determined by Folin-Ciocalteu reagent. There has been an increasing interest in identify natural antioxidant compounds to prevent the foods deterioration because of lipid oxidations and microbial spoilage, as well as to prevent harmful effects of free radicals on biological systems, Therefore, the aim of this experiment was to identify the best growth stage of *F. vaillantii* which has the highest antioxidant activity.

Materials and Methods

Plant materials

Aerial parts of *F. vaillantii* at different phenological stages (vegetative, budding and flowering) were collected from wild growing plants near to Torbat Heydarieh city in Northeastern of Iran with 2130 m altitude, 59° and 26" northern geographic latitude and 35° and 32" eastern geographic longitude, 235 mm annual rainfalls, minimum and maximum temperature of -20 and 38°C, respectively. A voucher specimen of the plant was deposited at the Herbarium of the Research Center for Plants Sciences, Ferdowsi University of Mashhad, Mashhad, Iran. During three growth stages (vegetative, budding, flowering), we selected three plants from each growth stage to evaluate total phenolic content and antioxidant activity.

Total phenolic content

Total phenolic content of the extracts were determined using the Folin-Ciocalteu reagent according to the method of Lowman and Box (1983), using gallic acid as a standard. Briefly, the solutions (0.1 mL) containing 1000 µg of the extracts were mixed with 46 mL of distilled water in a volumetric flask and 1 mL Folin-Ciocalteu reagent were added, and the flask was thoroughly shaken. The mixture was allowed to react for 3 min and then reached to 50 mL by 2% w/v of Na_2CO_3 solution. At the end of incubation of 2 h at room temperature, absorbance of each mixture

was measured at 760 nm. The same procedure was also applied to the standard solutions of gallic acid, and a standard curve was obtained. Total phenol contents were expressed as μg gallic acid equivalents per mg of the extract or the oil. All tests were carried out in triplicate, and gallic acid equivalent values were reported.

DPPH radical scavenging assay

This experimental procedure was adapted from Wang et al. (1998). In an ethanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical (final concentration was 1.0×10^{-4} M), test extracts were added, and their concentrations were 250, 500, 750, and 1000 μg/mL. The reaction mixtures were vigorously shaken and then were kept in the dark for 30 min. The absorbance of the resulting solutions was measured in 1 cm cuvettes, using a Jenway 6305 UV/VIS Spectrophotometer at 517 nm, against blank without DPPH. Decreasing of DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. This activity is given as %DPPH radical scavenging that is calculated from the following equation:

%DPPH radical scavenging = sample absorbance / control absorbance × 100

The DPPH solution without sample solution was used as control. All tests were run in triplicate and averaged. Ascorbic acid was used as positive control.

Ferric reducing antioxidant power (FRAP) assay

Antioxidant activity of extracts were determined according to the FRAP method that was previously described by Lado et al. (2004). To do FRAP assay, extract, (50 μL) were mixed with 1.5 mL FRAP reagent [25 mL of 300 mM acetate buffer, 2.5 mL of 10 m M 2,4,6-tri-2-pyridyl-2-triazine (TPTZ, Fluka, Sigma-Aldrich, Buchs, Switzerland) in 40 m MHCl and 2.5 mL of 20 mM $FeCl_3.6H_2O$], and incubated at 37 ºC for 5 min. The absorbance was measured at 594 nm using UV-visible spectrophotometer (Jenway 6305) against blank (FRAP reagent without the sample). The concentration of Fe^{2+}-TPTZ (reducing capacity) was calculated by comparing the absorbance at 594 nm with the standard curve of the Fe (II) standard solutions (ferrous sulfate heptahydrate).

Antioxidant activity by phosphomolybdenum complex method

The antioxidant activity of the *F. vaillantii* extracts was evaluated by the phosphomolybdenum complex method (Ghafoor and Choi, 2009). Briefly, 0.1 mL of sample solution (1 mg of *F. vaillantii* extract dissolved in 1 mL of ethanol) was combined with 1 mL of reagent solution containing 0.6 M sulfuric acid, 2 mM sodium phosphate, and 4 mM ammonium molybdate. The blank solution contained 4 mL of reagent solution and 1 mL of ethanol. Test tubes were capped and placed in hot water for 90 min at 95 °C. After samples were cooled to room temperature, absorbance was measured at 695 nm against a blank and the antioxidant activity was expressed as μmol BHT/g extract.

Statistical analysis

The experiment was carried out in triplicate, and data were subjected to ANOVA. ANOVA analyses were performed according to the SAS and figures were drawn by Excel software. Significant differences between means were determined by Duncan's multiple range tests ($P < 0.05$).

Results

Total phenolic content

Total phenolic content of ethanolic extracts were 68.38, 71.11 and 56.42 mg/g extract at vegetative, budding and flowering stages, respectively (Fig. 1). The lowest and highest phenolic contents were obtained at flowering and budding stages, although there was no significant difference between budding and vegetative stages.

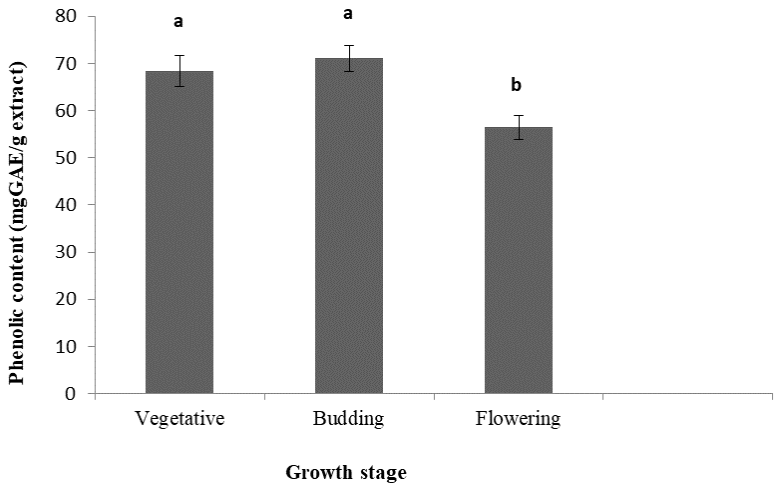

Fig. 1. Total phenolic contents of *Fumaria vaillantii* extracts at different phenological stages.

Table 1. Antioxidant activities of *Fumaria vaillantii* extracts at different phenological stages

Growth stage	DPPH IC_{50} (µg/mL)	FRAP (µmol Fe^{2+}/mg extract)	Phosphomolybdenum complex µM BHT/g extract)
Vegetative	1217.85±1.02 [c]	462.27±1.82 [b]	9496.70±236.71 [c]
Budding	1333.50±0.042 [b]	359.48±0.76 [c]	8481.10±182.40 [d]
Flowering	2577.10±1.75 [a]	248.87±0.66 [d]	18271.10±199.59 [a]
BHT	500.72±1.25 [d]	585.91±3.61 [a]	15056.66±346.46 [b]

Values in the table are means ± SD (Standard deviation). Different letters denote pairwise significant differences (ANOVA, $P \leq 0.01$) within each column

Antioxidant properties

- ***DPPH radical scavenging assay***

The antioxidant activity of *F. vaillantii* extracts were expressed as IC_{50}. A lower IC_{50} means better radical scavenging activity. There were significant differences among extracts and BHT ($P < 0.01$). The IC_{50} values of the samples increased from vegetative to flowering stages. Therefore, the highest antioxidant property (1217.85 ± 1.02) was observed at vegetative stage (Table 1).

Ferric reducing antioxidant power (FRAP) assay

The antioxidant activity of *F. vaillantii* extracts were reported by FRAP assay. Table 1 demonstrates that the reducing power of BHT (585.91 Fe^{2+}/mg extract) was significantly higher than vegetative, budding and flowering stages (426.27, 359.48 and 248.87 µmol Fe^{2+} per mg EO respectively). Among different growth stages of this plant, the highest antioxidant activity (462.27 ± 1.82) was observed at vegetative stage by this method.

Phosphomolybdenum complex method

The total antioxidant activity of ethanolic extracts of samples were investigated by using the phosphomolybdenum assay and expressed as BHT equivalents (mg BHT/g extract). The phosphomolybdenum method is based on the reduction of molybdenum (VI) to molybdenum (V) by extracts and the formation of a green phosphate/molybdenum (V) complex with a maximal absorption at 695 nm. The high absorbance values indicated that the sample possessed significant antioxidant activity. Flowering stage had the highest antioxidant activity (18271.10 µM BHT/g

extract), followed by BHT (15056.70 µM BHT/g extract) and vegetative stage (9496.70 µM BHT/g extract).

Discussion

Total phenolic content

Results from previous studies reported various quantities of phenolic compounds in ethanol extracts of fennel (Conforti et al., 2006), fenugreek seed (Ramya et al., 2011) and methanol extracts of *Fumaria vulgaris* (Jaberian et al., 2013). Variation in the amounts of phenolic compounds can be attributed to several reasons. Many of intrinsic factors such as plant species (genetic), parts of the plants and extrinsic factors such as environmental conditions (e.g. climate, soil, irrigation, temperature range, exposure to diseases and pests), cultural practices, harvest season, drying methods, handling and storage factors can influence the phenolic content of plants during the plant growth cycles (Tavassoli and Emam Djomeh, 2011). Regarding to the mentioned factor in this study we observed different phenolic contents in different growth stages. The plant extract composition can be changed with maturity. Therefore, the major components in early growth stage are different from flowering stage (Sellami et al., 2009). In accordance with our results, the highest phenolic content was observed at young stage of development in *Rosmarinus officinalis* (Del Baño et al., 2003). In this study, the maximum of total phenolic content obtained as maximum in vegetative stage but the lowest was in flowering stage. The highest accumulation amount of phenolic compounds was at the early growth stages may be because of great cellular division. Moreover, decrease in phenolic content with age is probably due to their dilution with growth (Wang et al., 2000; Del Baño et al., 2003). In previous researches the influence of age and seasonal variation in the concentration of phenolic compounds are reported (Munne´-Bosch et al., 2000; Sellami et al., 2009; Uddin et al., 2012). Phenolic compounds were exist during the earlier stages suggest a transport phenomenon toward the young organs. Some phenolic compounds disappeared from the vascular system by growing the plants. Endogenous biosynthesis and transportation in plants are two phenomena that can influence the distribution of phenolic compounds. A close relationship among different processes of biosynthesis, degradation, and transport are involved in the distribution of polyphenols in the plants (Del Baño et al., 2003).

Furthermore, the type of solvent (Cesoniene et al., 2012), the degree of polymerization of phenolics, interaction of phenolics with other food constituents and formation of insoluble complex are also important (Galvez et al., 2005). Recently, phenolic compounds have received considerable attention because of their physiological functions, including antioxidant and free radical-scavenging abilities that are affected quality and nutritional value (Govindarajan et al., 2007). Due to the previous reports, the phenolic compounds are associated with redox properties which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers as well as their metal chelating abilities (Viuda-Martos et al., 2010).

Antioxidant properties

There are many *in vitro* methods to assess the antioxidant activity, and depend on various generators of free radicals acting through different mechanisms to cover all aspects of antioxidant efficacy (Viuda-Martos et al., 2010), each method has its own advantages and limitations (Sanchez-Moreno, 2002). In this study, antioxidant activity of *F. vaillantii* extracts at different growth stages were evaluated by three methods including DPPH free radical scavenging, ferric reducing power (FRAP) and phosphomolybdenum complex methods. Also, BHT was used as reference or positive control.

DPPH is one of the most popular sensitive and frequently used methods for the determination of antioxidant activity of plant extracts (Fazal et al., 2011) which was usually explained with the presence of phenolic acids and flavonoids in them. Previously, strong antioxidant activity of methanol and methanol-water extracts of *Fumaria vaillantii*, determined by DPPH method (IC50 = 0.1273 and 0.1533 µg/mL extract, respectively) were reported (Jaberian et al., 2012). On the other hand, ethanolic extracts of *F. cilicica*, *F. densiflora*, *F. kralikii* and *F. parviflora* collected in Turkey, showed extremely low antioxidant activity in both DPPH and FRAP methods (Orhan et al., 2012). The antioxidant activity of methanolic extracts of fennel seeds (Anwar et al., 2009), *Foeniculum vulgaris* (Jaberian et al., 2012), and ethanolic extracts of fennel seeds (Anwar et al., 2009) and *Fumaria indica* (HabibiTirtash et al., 2011) were studied before which indicated a high effective free radical scavenging in the DPPH assay. The antioxidant activity of extract strongly depends on the extraction solvent which is generally selected according to the purpose of extraction, solvent concentration and extraction time which is influenced the extraction of functional components from plant materials (Wang et al., 2008), and compounds polarity as well as cost and safety (Yu et al., 2002). In agreement with our findings, variation in free radical scavenging activity has been observed during different plants growth stages. The effect of antioxidant on DPPH radical scavenging is due to their hydrogen donating ability which is accepted an electron or hydrogen radical to become a stable diamagnetic molecule (Hadbaoui et al., 2010). In addition, the reductive potential measures the ability of a sample to act as electron donor and, therefore, reacts with free radicals converting them to more stable products and thereby terminates radical chain reactions (Sarikurkcu et al., 2010). No data are available in the literature regarding the antioxidant activity of *F. vaillantii* with respect to the phenological stage variations in which to compare the results of present analysis.

FRAP test is an inexpensive procedure which is simple, reproducible and rapid, that measures the ability of antioxidant compounds to reduce the ferric ion Fe^{3+} to ferrous Fe^{2+}, as a measure of total antioxidant capacity (Prior and Cao, 1999). There is a direct correlation among the results that obtained from FRAP assay with DPPH and total phenolic content assay. Antioxidant activity is related to phenolic compounds that play a crucial role in neutralizing the free radicals as a result of the fact that phenolics have a hydroxyl group (Viuda-Martos et al., 2010). Partially similarities were found to exist among the results of the FRAP test and those of the DPPH radical-scavenging activity assay. According to the knowledge of authors there is no report on antioxidant activity of this plant to compare it with our findings.

The Phosphomolybdenum complex method is an alternative to the methods already available for the evaluation of antioxidant capacity due to its simplicity and the cheap reagents which it uses (Hadbaoui et al., 2010). The results of this method were in contrast to the DPPH and FRAP assays in this study, although there was no research on antioxidant activity of *Fumaria* species during growth stages and with various methods to compare with our results. It has been reported that the concentration of phenolic compounds can be affected by age and seasonal variation (Uddin et al., 2012). The plant extracts with high total phenolic content in Lamiaceae family and other plants such lemon balm, oregano, sage, dittany and hyssop have different applications as preservatives in the food and other industries (Spiridon et al., 2011; Skotti et al., 2014).

Previous studies showed close correlation between antioxidant capacities of medicinal plants and phenolic compounds from various natural sources (Verzelloni et al., 2007; Cesoniene et al., 2012) which are in accordance with our results in DPPH and FRAP assays, although differ from Phosphomolybdenum complex method. It is thus important to use several analytical methods and different substrates for measuring various characteristics of the antioxidant in order to evaluate the effectiveness of antioxidants. The DPPH method is less sensitive than the other methods for hydrophilic antioxidants, while FRAP is a simple test with a wide dilution range (Gil et al., 2000).

On the other hand, accumulation of secondary metabolites varies with the phenological stages of the plants (Omezzine and Haouala, 2013). According to the results of previous studies, the total phenolic content of extracts from fenugreek (Omezzine and Haouala, 2013) and *Asparagus racemosus* (Verma and Kasera, 2007) decreased from vegetative to fructification stages which are in accordance with the results of total phenolic content and antioxidant properties from DPPH and FRAP assays. In opposite with our findings, in has been shown that total polyphenolic content increased at the flowering stage in *Boerhavia diffusa, Sidacordifolia* (Verma and Kasera, 2007) and *Crithmum maritimum* (Males et al., 2003), during floral budding in *Hypericum hyssopifolium, H. scabrum* and at full-flowering in *H. pruinatum* (Ayan et al., 2007). Antioxidants have great importance regarding to reduce oxidative stress which could cause damage to biological molecules. Furthermore, natural antioxidant compounds have various mechanisms such as prevention of chain initiation and breaking by donating of hydrogen atoms or electrons, decomposition of peroxides, and prevention of continued hydrogen abstraction (Fazal et al., 2011).

Herbs contain free radical scavengers phenolic compounds that the most wide spread secondary metabolite as potential natural antioxidant due to their ability to act as both efficient radical scavengers and metal chelator (Chandrasekhar et al., 2006). Several factors including variety, plant parts, stage of maturity, postharvest and storage conditions as well as different process methods can influence the composition of antioxidants (Pandjaitan et al., 2005). The effects of senescence and aging on the antioxidant activity were previously reported (Page et al., 2000). For example, in spinach leaves higher levels of total phenolics and antioxidant capacity were reported at the mid-maturity stage in comparison with the immature stage (Pandjaitan et al., 2005). In addition, antioxidant activity increased with maturity in *Moringa oleifera* leaves (Sreelatha and Padma, 2009). The results in the present study showed that the antioxidants vary with the stage of maturity and analytical methods. From our results, it can be concluded that the best stage of *F. vaillantii* suited for consumption is the vegetative stage, when the maximum benefit of the antioxidant content can be derived.

Conclusions

The potential of antioxidant activity of extracts from many plants and medicinal natural sources in both fundamental science and food industries have gained increasing interest. Thus, it is important to increase the antioxidant intake in the diet and search for natural antioxidant sources among plants used as natural food additives or preservatives. Therefore, to substitute synthetic antioxidant by natural ones, studying the antioxidant capacity of plants is very important. The antioxidant activity of plant extracts should be evaluated in a variety of model systems using several different indices to ensure the effectiveness of such antioxidant materials. Iranian *F. vaillantii* at vegetative stage can be considered as a potential source of natural antioxidant for food and

pharmaceutical industries. The results obtained from this research clearly showed the antioxidant activity of *F. vaillantii* by various antioxidant methods. The results of this study indicated that total phenolic content and antioxidants activity were highest at vegetative stage. Therefore, it can be indicated that the best harvesting stage for obtaining high amount of natural antioxidants of *F. vaillantii*to use it in different industries such as pharmaceutical and food is vegetative stage.

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