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Antioxidant, Total Phenol and Flavonoid Contents of Two *Pedicularis* L. Species from Eastern Azerbaijan, Iran

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ABSTRACT

Introduction: *Pedicularis sibthorpii* and *P. wilhelmsiana* are endemic species mainly found in North-West of Iran. Plants of genus *Pedicularis* produce some important polyphenols and flavonoids. In the present work, total phenol and flavonoid contents of the mentioned species as well as their antioxidant capacity have been evaluated. ***Methods:*** Methanol extract of samples was fractionated by SPE method using an ODS cartridge and their ^{1}H-NMR spectra were recorded. Total phenols and flavonoids of methanol extracts were determined using Folin- Ciocalteu and aluminum chloride methods. For determining antioxidant activity of the extracts and fractions, bleaching of purple color methanol solution of 1, 1-diphenylpycryl hydrazyl (DPPH) was measured by spectrophotometric assay. ***Results:*** Total phenols of *Pedicularis sibthorpii* and *P. wilhelmsiana* were in the range of 8-30 mg g^{-1} and 9-20 mg g^{-1}, respectively. The 40% and 60% fractions of *P. sibthorpii* and the 20%, 40% and 60% fractions of *P. wilhelmsiana* showed higher amounts of phenolic compounds. The total flavonoid contents of *P. sibthorpii* and *P. wilhelmsiana* were in the range of 0-215 mg g^{-1} and 0-177 mg g^{-1}, respectively, whereas the 40% and 60% fractions showed higher flavonoid amounts. Antioxidant activity of *P. sibthorpii* and *P. wilhelmsiana* were in the range of 0.01-0.7 mg mL^{-1} and 0.01-1.02 mg mL^{-1}. In the same manner, the 20% and 40% fractions of *P. sibthorpii* and the 40% and 60% fractions of *P. wilhelmsiana* had lower RC_{50} than that of other fractions. ***Conclusion:*** Fractions with lower RC_{50} had higher contents of phenolic and flavonoid compounds. The results of NMR spectra were parallel with these findings and show that it is worth to do phytochemical studies on *P. sibthorpii and P. wilhelmsiana.*

Introduction

Free radicals can affect whole body system through contribution to many kinds of degenerative diseases including diabetes and cardiovascular damage (Yang *et al* 2010), CNS injury (Cadet 1998, Demopoulos *et al* 1982, Demopoulos *et al* 1980), cancer (Samra *et al* 2011, Vera-Ramirez *et al* 2011), liver and kidney damage (Muriel 2009, Small *et al* 2012), atherosclerosis, inflammatory joint disease, asthma (Rashidi *et al* 2010), gastritis (Salim 1992) and so on. Oxidation process is one of the most important routs for producing free radicals in food, drugs and even living systems (Mc Cord 2000).

Discovery of free radicals' impacts on biological system has led to the free-radical theory of aging which implies that preventing free radicals' action could influence lifespan. Though the healthy body produces a range of its own antioxidant to overwhelm free radicals, supplement natural antioxidants as free radical scavengers may be needed to boost the defensive system and slow down aging. This postulation has evoked great efforts for finding the powerful free radical scavengers to overcome harmful effects of oxidative stress. Recently attentions have been focused on the therapeutic potential of green foods and medicinal plants which is believed to reduce free radical induced tissue injury by trapping them (Beta *et al* 2005, Cai *et al* 2004, Chen *et al* 2007, Dudonne *et al* 2009). Higher plants produce a variety of antioxidant compounds of which, polyphenols are assumed to be the most potent one (Wang *et al* 2007, Guangrog *et al* 2008).

The genus *Pedicularis* produces iridoids, phenylethanoids, phenylpropanoids and flavonoids (Akdemir *et al* 1991, Fujii *et al* 1995, Liu *et al* 1991, Su *et al* 1998) which their antioxidant properties have been established, previously (Scalbert *et al* 2005). Majority of the *Pedicu-*

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laris species are widely distributed in China (Hanbi *et al* 1998) and most phytochemical studies have been carried out on these species. *Pedicularis* species have different therapeutic applications in Traditional Chinese Medicine (Jiang *et al* 2003, Zhang *et al* 2008). As part of our ongoing research on bioactive constituents of Iranian medicinal plants, we focused on native *Pedicularis* species. *Pedicularis* genus is represented by 9 species in flora of Iran (Wendelbo 1981) which their medicinal uses are unknown. *Pedicularis sibthorpii* and *P. wilhelmsiana* grow in Azerbaijan province, Iran and according to the best of our knowledge, their biological effects and chemical constituents are not investigated, yet. In the present work, the results of DPPH assay, total phenol and flavonoid content of *P. sibthorpii* Boiss and *P. wilhelmsiana* Fisch. extracts and their relationship with ^{1}H-NMR spectra have been reported.

Materials and methods

Plant materials

The samples of *Pedicularis sibthorpii* Boiss. were collected from Lighvan valley -Tabriz and samples of *P. wilhelmsiana* Fisch. ex M. Bieb. were collected from Arasbaran region in East Azerbaijan province, Iran in 2009. Voucher specimens for these collections (*P. wilhelmsiana*, TUM- FPh 700; *P. sibthorpii*, TUM- FPh 701) have been deposited in the Herbarium of the Faculty of Pharmacy, Tabriz - Iran.

Extraction and fractionation

Powdered air-dried aerial parts of plants (200g) were extracted by n-hexane (8 h), dichloromethane (10 h) and methanol (8 h) using a Soxhlet, respectively. Solvents were removed *in vacuo* by rotary evaporator at an ambient temp. Further studies were carried out on methanol extract fractions prepared by solid phase extraction (SPE) method.

The methanol extract (2 g) was loaded on a Sep-pak (Vac 35 cc; 10g; C18 cartridge) cartridge and fractions were eluted by step gradients of methanol- water mixtures (fr1,10% methanol; fr2, 20% methanol; fr3, 40% methanol; fr4, 60% methanol; fr5, 80% methanol; fr6, 100% methanol; 200 mL each). The solvents of eluted fractions were removed in vacuo and 40°C.

DPPH assay

Antioxidant activity of the extracts and fractions was measured according to Asnaashari *et al* (2011) with some modifications. In order to obtain dilutions, different sample concentrations were prepared in methanol and 5 mL of each concentration were added to 5mL of 0.004% methanolic solution of DPPH. After completion of reaction at room temperature for 30 min, bleaching of DPPH was monitored at 517 nm against a blank. Inhibition of DPPH was calculated as RC_{50}, extrapolated from dose-response curve. Tests were carried out in triplicate and the same procedure was applied for the quercetin as a positive control.

Determination of total phenol content

Determination of total phenol content was carried out by Folin- Ciocalteu test according to Chun *et al* (2003) with some modifications. One mL of the extract and fractions' sample (solved in 60% acetone, 5 mg/100 mL) was mixed with 200 µl Folin- Ciocalteu reagent and 1 mL of aqueous Na_2CO_3. The mixtures were left at room temperature for 30 min and the phenol contents were determined by colorimetric method at 715 nm. The calibration curve was prepared using Gallic acid solutions at concentrations of 1- 0.01562 mg/mL in 60% acetone. Total phenol contents were expressed in terms of gallic acid equivalent (mg g^{-1}).

Determination of total flavonoids content

The total flavonoid content was estimated using aluminum chloride colorimetric assay (Zhishen *et al* 1999, Zou *et al* 2004). The 0.5 mL of test samples' solution in methanol (5mg/100mL) were mixed with 2mL of distilled water and 150 µl of 5% sodium nitrate. After 6 min, 150 µl of 10% aluminum chloride and 2mL of 1 M sodium hydroxide were added and left at room temperature for 15 min. Absorbance of the mixtures was measured at 510 nm (UV-Visible Ultraspec 2000 spectrophotometer, England) and total flavonoid contents were calculated as rutoside equivalents from a calibration curve of rutoside. The calibration curve was prepared in the same manner using 0.01562-1 mg/mL of rutoside solutions in methanol.

NMR spectra from methanolic fractions

NMR spectra were recorded in CD_3OD on a Bruker 200 MHz NMR spectrometer. TMS was used as the internal standard.

Results and discussion

According to the literature records, chemical constituents and bioactivities of different *Pedicularis* species have previously been investigated. Iridoids, phenylethanoids, phenylpropanoids and flavonoids are the major secondary metabolites of the genus *Pedicularis* (Akdemir *et al* 1991, Fujii *et al* 1995, Liu *et al* 1991, Su *et al* 1998). The antioxidant effects of phenylpropanoid glycosides, widely distributed in genus *Pedicularis*, were studied in different models (Zheng *et al* 1993, Scalbert *et al* 2005, Hosoya *et al* 2008, Ahmad *et al* 2009, Biao *et al* 2009, Zhu *et al* 2010).

Polyphenols consisting of a wide range of biogenic molecules play numerous roles in living organisms. Flavonoids, as the main class of polyphenols, widely distri-

buted in plants, display numerous pharmacological effects (Rashidi *et al* 2010). It has been shown that free radical scavenging property of flavonoids is responsible for these effects (Cotelle *et al* 1996, Brown *et al* 1998, Seyoum *et al* 2006, Tsimogiannis *et al* 2006). A common and rapid method to evaluate free radical scavenging ability of flavonoids involves the use of stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Extracts of *P. sibthorpii* and *P. wilhelmsiana* and their fractions exhibited varying degrees of free radical scavenging capacities as determined by DPPH methods (Table 1 and Table 2). Total extract of *P. sibthorpii* (RC_{50}= 0.033 mg mL^{-1}) showed stronger radical scavenging effect than that of *P. wilhelmsiana* (RC_{50}= 0.15 mg mL^{-1}). Considering the RC_{50} of quercetin (2.92 x 10^{-5} mg mL^{-1}), it could be a moderate effect. The experiments showed that the 20% and 40% Sep-Pak fractions of *P. sibthorpii* as well as the 40% and 60% Sep-Pak fractions of *P. wilhelmsiana* have lower RC_{50} than that of other fractions.

Total phenols were assessed by Folin Ciocalteu method and results were reported as gallic acid equivalents by reference to standard curve (y =0.0282+0.0043, r^2=0.998). Total phenols of *P. sibthorpii* and *P. wilhelmsiana* and their fraction were demonstrated in Table 1 and Table 2. According to these findings, 40% and 60% Sep-Pak fractions revealed the highest amount of total phenols at 30.06 and 25.07 mg g^{-1} respectively, approximately 2 times more than methanol extracts.

Flavonoid content was determined by aluminum chloride method and expressed as rutoside equivalents in mg g^{-1} dry extracts and fractions using a standard curve of rutoside (y= 2.0903x- 0.0298, r^2= 0.998). As can be seen from the Table 1 and Table 2, flavonoid content of 40% and 60% Sep-Pak fractions was superior to the flavonoid content of other fractions and extracts. The lowest flavonoid content was found within 20%, 80% and 100% fractions.

The collected data have shown the existence of a good harmony between antioxidant potency of samples and their total phenolic and flavonoid content. The maximum antioxidant, total polyphenols and flavonoid content were seen in 40% and 60% Sep-Pak fractions whereas the other fractions were found to contain nearly low or neglectable amounts. Generally, the higher antioxidant activity of the mentioned fractions might be attributed to their contents of total polyphenols and especially flavonoids. However according to Prior *et al* (2005), many other compounds may contribute to the reaction and cause a false positive error.

In the present study, the issue was partly clarified by using ^{1}H- NMR spectra as fingerprints to indicate significant differences between the chemical constituents of fractions (Figures 1 and 2). The ^{1}H- NMR spectra of 10% Sep-Pak fractions belonging to *P. sibthorpii* and *P. wilhelmsiana* (Figures 1-a and 2-a, respectively) have revealed that there is no phenolic compound, but iridoids (signals in the δ 3-6 ppm) in this fraction. In the same way, the Figures 1-b and 2-b show that some phenolic compounds such as phenylethanoids and flavonoids may exist in low concentrations in 20% fractions of *P. sibthorpii* and *P. wilhelmsiana*. Verbascoside is a common phenylpropanoid of the *Pedicularis* genus and its signals have clearly been seen in the ^{1}H-NMR spectra of 40% fraction (Figures 1-c and 2-c). Flavonoids may exist in 40% fractions in lower concentration, but their signals were covered by verbascoside signals due to its high concentration. Figures 1-d and 2-d, which are NMR spectra of 60% fraction, indicate that phenolic compounds such as phenylethanoids and flavonoids exist in this fraction. Obviously phenolic compounds exist in very low concentrations in 80% fractions (Figures 1-e and 2-e) and there is no sign of similar compounds in 100% fractions (Figures 1-f and 2-f).

According to these finding, it can be concluded that there is a relationship between antioxidant activity, phenolic and flavonoid content in these two species, because fractions with lower RC_{50} have higher contents of phenolic and flavonoid compounds. The results of NMR spectroscopy are parallel with these findings, too. Fractions which show signals in aromatic regions (δ 6-8 ppm) possess higher polyphenolic compounds and lower RC50.

Table1. Antioxidant, total phenol and flavonoid contents of *P. sibthorpii*

Extract or fractions	Total phenol content (as gallic acid equivalents) mg g^{-1}	Flavonoid content mg g^{-1}	Antioxidant activity[a] (RC_{50}; mg mL^{-1})
Total extract	15.28	47.50	0.0330
10%	08.56	01.80	00.820
20%	12.59	00.00	00.070
40%	30.06	118.61	00.012
60%	25.07	214.76	00.107
80%	10.74	00.00	00.337
100%	10.01	00.00	00.616

[a] Positive control (quercetin): 2.92 x 10^{-5}

Table 2. Antioxidant, total phenol and flavonoid contents of *P. wilhelmsiana*

Extract or fractions	Total phenol content (as gallic acid equivalents) mg g^{-1}	Flavonoid content mg g^{-1}	Antioxidant activity[a] (RC_{50}; mg mL^{-1})
Total extract	12.18	35.60	00.15
10%	09.17	01.55	03.82
20%	17.52	00.00	00.18
40%	19.31	154.14	0.017
60%	18.43	176.60	00.0740
80%	11.32	00.00	00.1774
100%	09.84	00.00	01.0192

[a] Positive control(quercetin): 2.92 x 10^{-5}

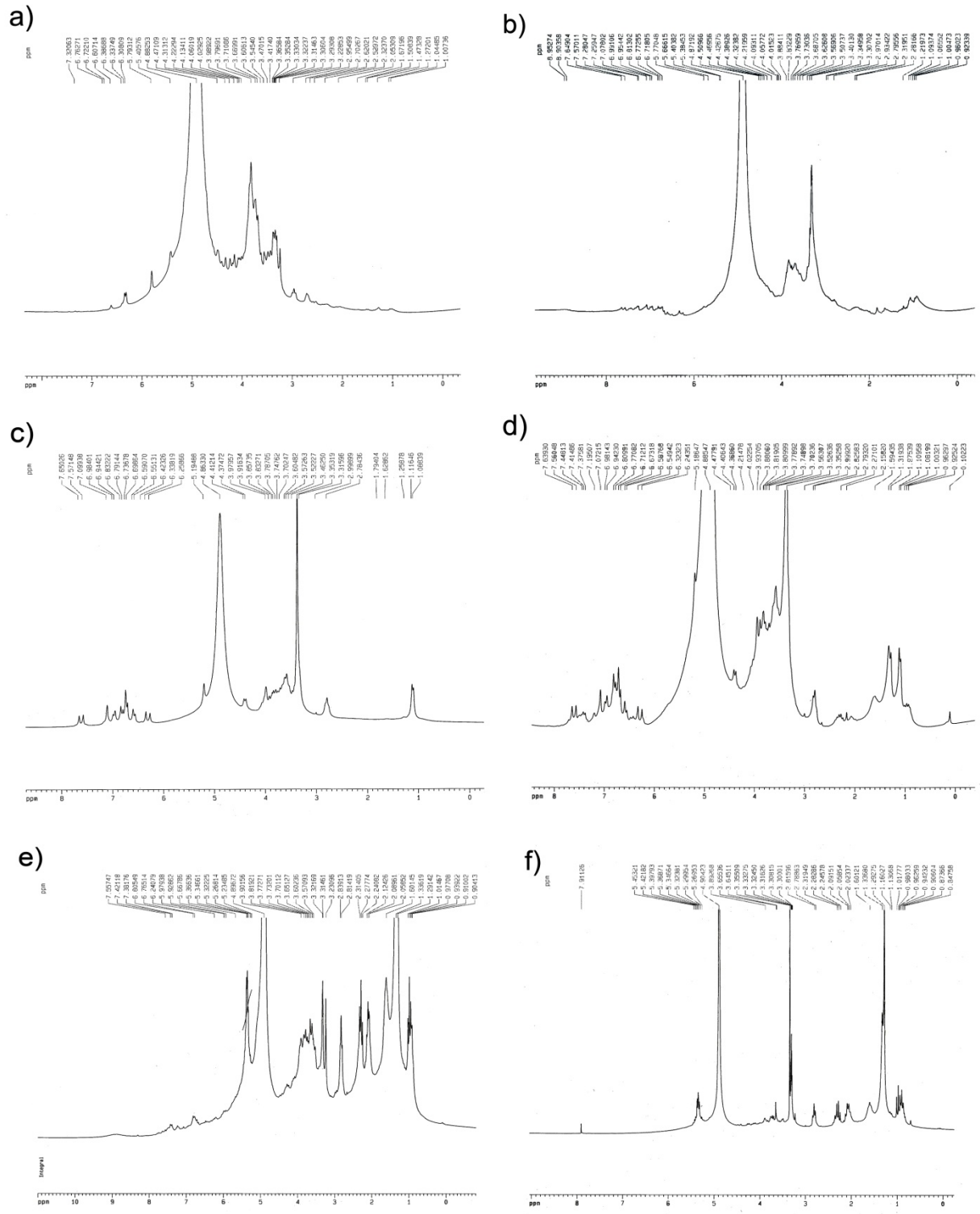

Fig. 1. [1]H-NMR spectra of Sep-Pak fraction obtained from *P. sibthropii* methanolic extract. The spectra were recorded in CD3OD. a) 10% fraction, b) 20% fraction, c) 40% fraction, d) 60% fraction, e) 80% fraction and f) 100% fraction.

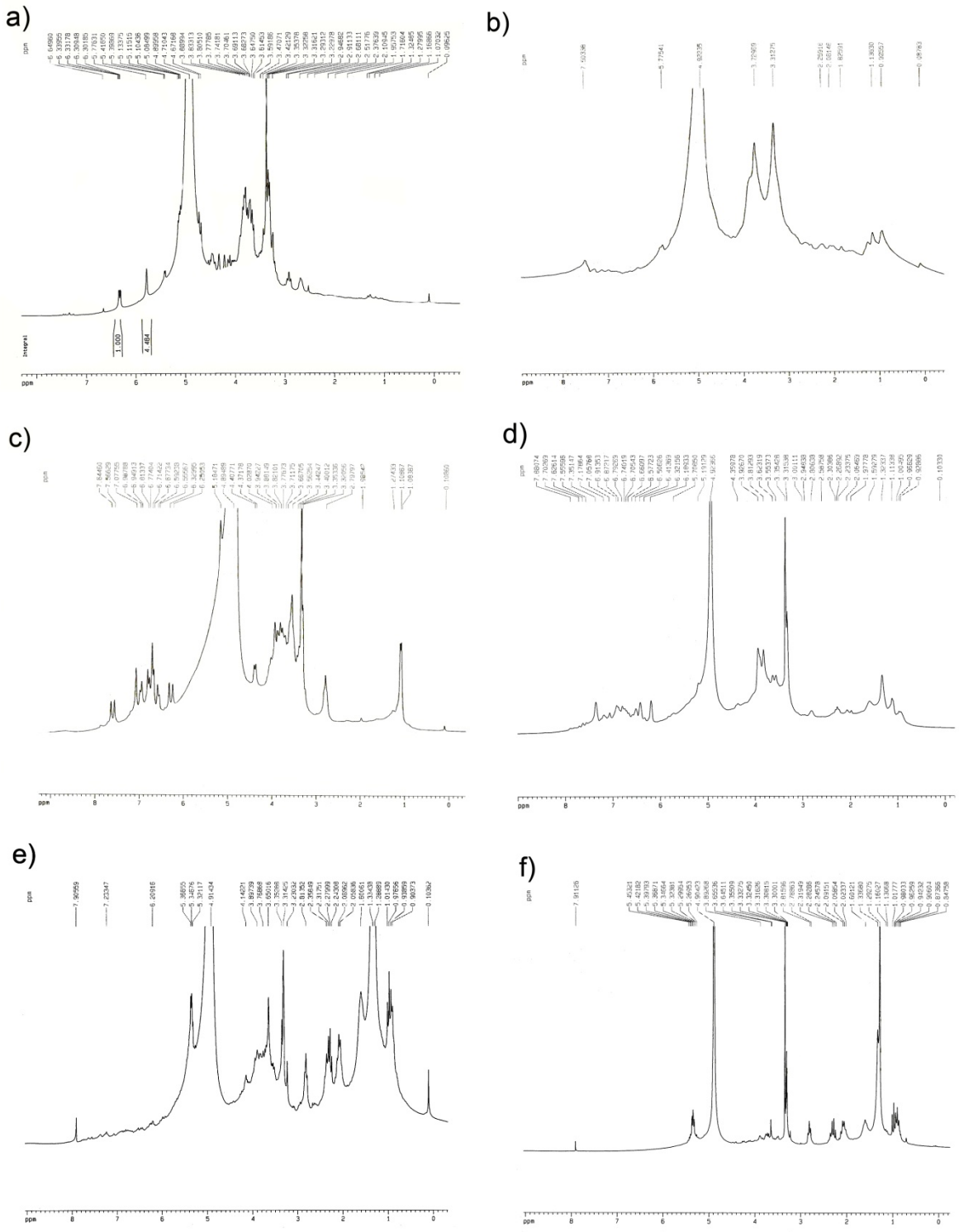

Fig. 2. ^{1}H-NMR spectra of Sep-Pak fraction obtained from *P. wilhelmsiana* methanolic extract. The spectra were recorded in CD_3OD. a) 10% fraction, b) 20% fraction, c) 40% fraction, d) 60% fraction, e) 80% fraction and f) 100% fraction.

Conclusion

Increasing demands for natural antioxidant have provoked great efforts for finding new and potent free radical scavengers from plants. *Pedicularis* species yield a series of antioxidant, antitumor, antifungal, antidiabetic and anti-inflammatory secondary metabolites (Beta *et al* 2005, Cai *et al* 2004). According to some evidences, phenylpropanoids and flavonoids are responsible for antioxidant effects in plants (Choudhary *et al* 2011).

The results of present work show that it is worth to do phytochemical studies on Iranian *Pedicularis* species and purify their antioxidant compounds which are estimated to be phenylethanoids and flavonoids according to the ^{1}H-NMR findings. It could be concluded that fractionation of extracts and running their H-NMR could be a valuable method for predicting and comparing their antioxidant capacity.

Ethical issues

Not applicable in this research.

Conflict of interests

Authors declared no conflicts of interests.

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