Evaluation of Total Phenolic and Flavonoid Content, Antioxidant Capacity and Resveratrol of Selected Medicinal Plants of Northern Iran

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Summary

Medicinal plants are source of bioactive molecules with antioxidant activity, which are well-known due to their health effects. Identification and quantitation of these compounds helps to evaluate the therapeutic properties of medicinal herbs. In this study total phenolic and flavonoid content, antioxidant capacity and resveratrol in fourteen species were estimated using Folin-Ciocalteu method, aluminium chloride colorimetric assay, DPPH radical scavenging capacity and liquid chromatography, respectively. Among plant materials the highest total phenolic was observed in Iranian barberry (*Berberis integerrima* Bunge.) and the highest total flavonoid was recorded in persicaria bistorta (*Polygonum bistorta* L.), while remarkable high antioxidant capacity was noticed in small-flowered black hawthorn (*Crataeagus pyntagyna* Waldst. & Kit. ex Willd). Wild grape (*Vitis vinifera* L. ssp. *silvestris*) was the richest source of *trans*-resveratrol (14.0±0.90 mg/100g DW). Further studies of these herbs would help knowing pharmaceutical potential of the northern Iran plants.

Key words

medicinal plant, flavonoid content, antioxidant capacity, resveratrol, phenolic, Iran

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Introduction

Medicinal herbs that are rich sources of phytochemicals have an important role in ensuring human health. Basically, it is necessary to determine secondary metabolites in the medicinal plants. Phytochemicals play an important role in scavenging oxygen radicals and in protecting the consumers against diseases. They could be used to treat the various biotic and abiotic disorders. Meanwhile, phenolic compounds and flavonoids play important physiological and biochemical role in plants such as in their appearance and taste (Tapas et al., 2008). These substances may be in whole plants or parts of plants such as fruits, seeds, leaves, roots and barks (Atanassova et al., 2011).

Resveratrol (3,5,4′-trihydroxystilbene) is a phytoalexin with a protective antibiotic role. It is assumed to be produced in plants under several stresses caused by fungal attack, drought, and ultraviolet irradiation (Hasan et al., 2013; Romero-Pérez et al., 2001). It has been reported in more than 70 plant species such as itadori tea, red grape, raspberry, mulberry, plum, peanut, bilberry, blueberry and cranberry (Deng et al., 2012; Fan et al., 2011), among which itadori tea and red wine are the main sources for resveratrol isolation (Burns et al., 2002; Kiselev, 2011). It was demonstrated that resveratrol has protective effect against numerous disorders namely cancer, diabetes, Alzheimer, and cardiovascular and pulmonary diseases (Bhat et al., 2001; Deng et al., 2012; Fan et al., 2011; Frémont, 2000). Resveratrol is in forms of two structural isomers: cis and *trans*. The *trans*-isomer is the more stable and biologically active form. Both of the isomers can be present in different amounts in plants, but the amount of *trans*-resveratrol usually predominates (Neves et al., 2012). The determination of resveratrol in the real matrices usually requires utilization of separation methods. Among currently used methods, the high-performance liquid chromatography (HPLC) coupled with various detectors, gas chromatography-mass spectrometry (GC-MS) and capillary electrophoresis (CE) are the mostly beneficial (Fan et al., 2011).

Iran is a well-known origin of genetic diversity of medicinal plants and its Guilan province is a rich area of plants containing resveratrol. Hence, this study was planned to evaluate some plant materials collected from various regions of Guilan in order to find new potential sources of natural antioxidants.

Materials and methods

Plant material and chemicals

Samples of 14 medicinal plants were collected from the different regions of Guilan province in 2016 (Table 1), and dried in hot air oven at 40°C for 72 h. *Trans*-resveratrol standard (purity > 97%) was purchased from Sigma-Aldrich Chemical Co. All reagents such as DPPH, Folin-Ciocalteu were purchased from E-Merck and other materials such as solvents (methanol, ethanol, acetic acid, acetonitrile, ect.) and chemicals (gallic acid, catechin, aluminum chloride, ect) obtained from Sigma-Aldrich Chemical Co.

Sample extraction

One gram of specimen was ground up and then 3 mL methanol: acetic acid (85:15, v/v) was added and shook overnight on a shaker at 100 rpm at room temperature. Sample was incubated for 24 h at 4°C. Then, mixtures were centrifuged at 10000 rpm for 15 min and supernatant fluids were stored at -20°C until analysis (Bakhshi and Arakawa, 2006).

Total phenolic content

Total phenolic content (TPC) was measured using the Folin-Ciocalteu colorimetric procedure based on the formation of a blue molybdenum-tungsten complex. Gallic acid was used as standard (Guerrero et al., 2011). Samples were diluted accordingly, 50 µL of the methanolic extract was mixed with 150 µL of distilled water (DI) in a test tube followed by addition of 1 mL of 10% Folin–Ciocalteu reagent and kept for 6 min. 800 µL of 7.5% sodium carbonate solution was added to the test tubes. Samples were kept for 90 min at room temperature in darkness (Lister et al., 1994). The absorbance was measured at 760 nm with UV-VIS spectrophotometer (PG Instrument +80 Leicester, UK). Results were expressed as mg gallic acid equivalent per 1g of dry sample (GAE/1 g DW).

Total flavonoid content

Total flavonoid content (TFC) of samples was determined based on the aluminum chloride colorimetric procedure with some modifications (Park et al., 2008). First, samples were diluted 1/5. In a 2 mL test tube, 100 µL of extract, 1750 µL of DI, 75 µL of $NaNO_2$ (0.5 M) and 75 µL of $AlCl_3.6H_2O$ (0.3 M) were mixed. 500 µL of NaOH (1 M) was added after 5 min. The solution was shaked well and absorbance of the mixture was determined at 506 nm. Catechin standard procedure of 0 to 120 mg/l was prepared as standard curve. Flavonoid content was expressed as mg catechin equivalent per 1 g dry weight (CAT/1g DW).

Table1. The samples of 14 medicinal plants collected from the different areas of Guilan province, Iran

Name	Family	Locality	Edible parts analyzed
Morus alba L.	Moraceae	Bandar-e Anzali	Root, leaf
Polygonum bistorta L.	Polygonaceae	Bandar-e Anzali	Root
Cornus mas L.	Cornaceae	Rezvanshahr	Fruit skin (Exocarpe)
Eucalyptus globulus Labill.	Myrtaceae	Rezvanshahr	leaf
Crataegus microphylla C. Koch.	Rosaceae	Talesh	Fruit skin (Exocarpe)
Crataegus pyntagyna Waldst. & Kit. ex Willd.	Rosaceae	Talesh	Fruit skin (Exocarpe)
Arachis hypogaea L.	Fabaceae	Astaneh-e Ashrafiyyeh	Seed skins (peanut shell)
Vitis vinifera L.	Vitceae	Rudsar	Fruit skin (Exocarpe)
Vaccinium arctostaphylos L.	Ericaceae	Talesh	Fruit skin (Exocarpe)
Elaeagnus umbellate Thunb.	Elaeagnaceae	Rasht	Fruit skin (Exocarpe)
Vitis vinifera L. ssp. silvestris	Vitaceae	Rudsar	Fruit skin (Exocarpe)
Berberis integerima Bunge.	Berberidaceae	deylaman	Fruit skin (Exocarpe)
Rumex asetosella L.	Polygonaceae	Rasht	Root
Rubus hyrcanus L.	Rosaceae	Bandar-e Anzali	Fruit skin (Exocarpe)

Antioxidant capacity

The antioxidant capacity (AOX) was evaluated by DPPH (2,2'-diphenyl-1-picrylhydrazyl) free radical scavenging assay using the method described by Brand-Williams and coworkers, with some modifications (Brand-Williams et al., 1995). 50 µL of the sample extracted was added to 950 µL of DPPH radical. The solution was vortexed and allowed to stand at room temperature in darkness. The absorbance of the samples was assayed at 517 nm after 15 min. The percentage of DPPH radical scavenging capacity was calculated according to the following equation:

DPPH scavenging effect (%) = ((A0 –A1)/A0)) × 100

Where; A0 is the absorbance of control DPPH solution at 0 min and A1 is the absorbance in the presence of test sample at 15 min.

Trans- resveratrol content (TRC)

The resveratrol analysis was carried out by a reversed phase high-performance liquid chromatography (HPLC) system (Waters, 1525, Milford, USA) equipped with a UV-Visible detector (Waters Dual λ Absorbance 2487). Detection was monitored at 306 nm. The column was a Symmetry C18 (4.6×150 mm, 5 µm; Waters, Dublin, Ireland). The mobile phase was HPLC grade acidified water containing 3% acetic acid (A) and acetonitrile (B). The gradient program was: B: 0.00 –5.00 min, 0–8.5%; 5.00–16.50 min, 8.5–2.0%;16.50–35.00 min, 2.0–18%; 35.00–50.00 min, 18–20%; 50.00–65.00 min, 20–30%; 65.00–70.00 min, 30–0%. The column was held at 25°C and flushed at a flow rate of 1 mL/min. Detection, quantification and calculation of *trans*-resveratrol were accomplished using the external standard. Standard procedure was established by injection of 0.25, 0.125, 0.062 and 0.031mg/l. Chromatographic identification and confirmation of *trans*-resveratrol were based on comparing retention times with the authentic standard and on-line UV absorption spectrum data (Zhang et al., 2011).

To 1 gr of sample was added 10 mL 1 M HCl/methanol/water, 1/80/19, v/v/v, and shaken overnight on a shaker at 100 rpm at room temperature. The aqueous phase was poured in a 250 mL flask and concentrated in a rotary evaporator (R 2119, Heidolph) at 35°C to a volume of 2 mL. The concentrated sample was centrifuged at 10 000 rpm for 10 min. The supernatant fluids were filtered through disposable 0.45 µm syringe filter and stored at -20°C until analysis (Zhang et al., 2011).

Statistical analyses

Obtained data were analyzed on the basis of a completely random design with three replicates for each sample and by ANOVA. Means were compared with the Duncan's multiple range test ($p \leq 0.05$). A correlation analysis and linear regression were applied to all indices here, using SAS software (Ver. 9.1).

Results

These results revealed that the examined species are significantly different ($p<0.01$) in all evaluated phytochemical characteristics including TPC, TFC, AOX and TRC (Table 2).

Total phenolic, flavonoid content and antioxidant capacity

The total phenols varied in the plant materials from 2.3 to 31.2 mg/g GAE. The lowest TPC was found in autumn olive (*Elaeagnus umbellate* Thunb.), whereas the highest was found in Iranian barberry (*Berberis integerrima* Bunge). TFC widely varied among the

Table 2. Analysis of variance with mean squares for TFC, TPC, AOX and TRC of the extracts as affected by species

Parameter	Source	df	Mean squares	CV	P-value
TPC	Species	14	230.1819	2.2	< 0.01
	Error	30	0.093249		
TFC	Species	14	66.88023	2.8	< 0.01
	Error	30	0.031213		
AOX	Species	14	470.2277	0.7	< 0.01
	Error	30	0.141713		
TRC	Species	14	0.002331	16	< 0.01
	Error	15	$9.29*10^{-6}$		

Table 3. Total phenol content, total flavonoid content, antioxidant capacity and resveratrol content of the 15 species

Plant material	TPC (mg GAE/g dw)	TFC (mg CTE/g dw)	AOX (%)	TRC (mg/100g dw)
Morus alba, root	8.5±0.07 ij	4.4±0.07 g	60.6±0.57cd	0 f
Morus alba, leaf	2.4±0.04 l	1.9±0.11 j	28.3±0.10 k	1.5±0.10 d
Polygonum bistorta	15.4±0.09 f	14.7±0.39 a	49.4±0.15 h	0 f
Cornus mas	3.1±0.05 k	0.5±0.02 k	60.3±0.14 d	0. 4±0.03 ef
Eucalyptus globulus	15.1±0.03 f	4.3±0.18 g	62.6±0.06 b	0 f
Crataegus microphylla	12.5±0.09 g	8.5±0.09 f	61.0±0.28 c	0. 5±0.02 ef
Crataegus melano	20.2±0.06 d	12.3±0.23 c	65.4±0.11 a	0. 7±0.04 e
Arachis hypogaea	8.0±0.04 j	3.5±0.05 h	60.9±0.2 cd	8.4±0.70 b
Vitis vinifera	9.4±0.06 h	4.5±0.17 g	28.3±0.07 k	3.2±0.40 c
Vaccinium arctostaphylos	21.1±0.05 c	9.7±0.24 d	53.1±0.05 f	3.4±0.50 c
Elaeagnus umbellata	2.3±0.01 l	0.3±0.05 k	37.4±0.12 j	0. 2±0.04 ef
Vitis sylvestris	8.8±0.23 i	2.9±0.05 i	50.5±0.08 g	14.0±0.90 a
Berberis integerima	31.2±0.14 a	13.6±0.34 b	56.3±0.14 e	0. 8±.06 e
Rumex asetosella	26.8±0.07 b	3.4±0.05 h	62.6±0.09 b	0. 5±0.03 ef
Rubus hyrcanus	18.7±0.23 e	9.1±0.21 e	40.9±0.37 i	0 f
Correlation				
TPC	-	0.7**	0.4*	-0.1^{ns}
TFC	-	-	0.16^{ns}	-0.2^{ns}
AOX	-	-	-	0.02^{ns}

Different letters among species based on the treatment indicate a significant difference according to Duncan s multiple range test (P ≤ 0.05). Each value is expressed as the mean ± standard error (n = 3). Ns, non-significant; ** Correlation is significant at the 0.01 level; * Correlation is significant at the 0.05 level

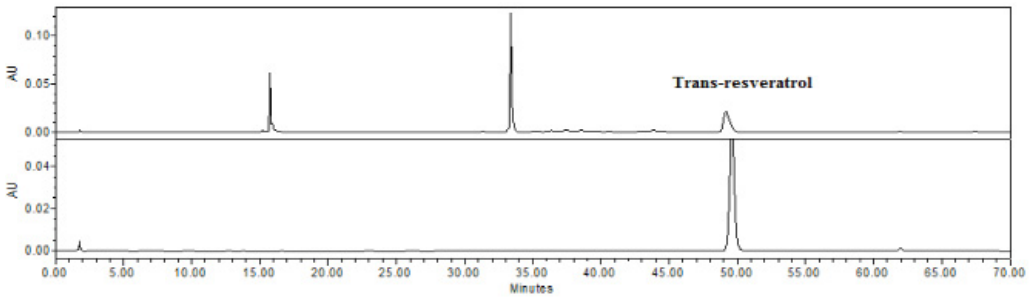

Figure 1.
HPLC chromatogram obtained from wild grape extracts (top) and *trans*- resveratrol standard (bottom) captured at 306 nm

samples and ranged from 0.3 to 14.7. Like before, lowest TFC were found in autumn olive while the highest TFC was found in extract of bistort (*Polygonum bistorta* L.). The AOX ranged from 28.3 to 65.4%. The highest value of AOX was obtained from small-flowered black hawthorn (*Crataeagus pyntagyna* Waldst. & Kit. ex Willd.), and the lowest AOX was recorded in leafs of white mulberry (*Morus alba* L.) and grape (*Vitis vinifera* L.) (Table 3)

***Trans*-resveratrol content**

The HPLC results demonstrated that wild grape (*Vitis vinifera* subsp. *sylvestris*) had the highest *trans*-resveratrol (14.0±0.90 mg/100g D.W). The lowest TRC was obtained in autumn olive. The results revealed that resveratrol was not found in four samples (Table 3). The retention times of *trans*- resveratrol was 48 min in our analysis conditions (Figure 1).

Relationship between TPC, TFC, AOX and TRC

There was a positive correlation between total phenolic content, antioxidant capacity (r = 0.7) and flavonoids content (r = 0.4), but not with resveratrol. There was a weak correlation between total flavonoid content and antioxidant capacity (r= 0.2) (Table 3).

Discussion

Phenolic compounds are found in a wide variety of plants. Furthermore, the phenolic accumulation is highly related to the level of stress factors (Dixon and Paiva, 1995). Study in northern Greece, showed that native Cornelian cherry (*Cornus mas* L.) was the richest source of antioxidants among the studied herbs (Pantelidis et al., 2007). In the current study, Cornelian cherry had a high antioxidant capacity but not the highest. This could be due to differences in climate and method of antioxidant capacity evaluation; as they used FRAP (ferric reducing ability of plasma) method using fresh sample. Results here confirm a variation in phenolic and flavonoid content among studied materials. Moreover, Iranian barberry is a rich source of phenol and flavonoid. Bayani et al. (2016) reported that Iranian barberry had the highest phenolic content (6.816 ± 0.132 mg/g dw) while Ardestani et al. (2013) recorded 8530 mg in 100 g of fresh fruits. These differences may be due to differences in climate factors and the method of measurement. In agreement with the current work, it is reported that bistort is considered as the source of valuable biologically active compounds such as phenolic acids, flavonoids, triterpenoids and steroids (Voronkova and Vysochina, 2014). The Results reported by Samec and Piljac-Jasenka (2011) revealed that hawthorn exhibited the high initial antioxidant capacity using FRAP (6.33 ± 0.10 mmol Fe2+/100 g FW). Previous studies have demonstrated the high phenolic and flavonoid compounds could be responsible for antioxidant activity and pharmacologic actions in black hawthorn, which had high anti-radical activity against DPPH, but some studies emphasize that this property could associate with high level of vitamin C in this plant (Yoo et al., 2008). Our results are in accordance with those reported by other scholars (Bedreag et al., 2014; Liu et al., 2010; Rabiei et al., 2012; Samec and Piljac-Zegarac, 2011; Zhang et al., 2001). There was a positive correlation between phenol and flavonoid content and the mild correlation with antioxidant capacity but not with resveratrol. From previous studies, a linear relationship was reported between antioxidant activity and total phenolic content in the selected spices (Cai et al., 2004; John et al., 2014; Kahkonen et al., 1999; Katalinic et al., 2006; Singh, 2015; Tukun et al., 2014). There was no significant correlation between flavonoid content and antioxidant capacity, therefore the flavonoids in the herbs do not influence their potential of DPPH radicals scavenging as reported by other researchers (Barcelo, 2015; Miliauskas et al., 2004; Nickavar and Abolhasani, 2009).

Lack of correlation between phenol and resveratrol content and weak correlation between total phenolic and antioxidant capacity must be due to the compounds not measured here. It is well known that phenolic compounds are mainly responsible for antioxidant activities. However, phenolic compounds will be a tiny fraction of the total antioxidant capacity. Also, the biochemical method of quantification for total phenol and flavonoid are roughly proportional based on the type of phenolic compounds, number of hydroxyl groups and their position. Resveratrol, a stilbene compound, is a minor phenolic compound.

Although, the observed antioxidant capacity might not to be solely from the phenolic content, it could possibly be due to the presence of some other phytochemicals that showed the synergistic effects. This synergistic effect might also contribute to the antioxidant capacity of plant extract (Bajpai et al., 2005; Kähkönen et al., 1999; Kaur and Mondal, 2014).

Results here showed that wild grape was the richest source and peanut was the second-best source of resveratrol. The resveratrol, piceid and piceatannol stilbene content of 21 Italian red grapes are reported to range from 19 to 508 µg/g (Vincenzi et al., 2013) that was almost near to the total stilbenes observed in wild grape in the current research.

In the study on 86 genotypes of wild grape, two clusters with high potential of stilbenes production were highlighted. Peanut shell was introduced as the commercial source for resveratrol-full extract after the grape and the wine (Hasan et al., 2013). For the lack of resveratrol in four samples here, it could be said that environment, genetic factors, developmental stages and stress agents has effect on resveratrol production (Bavaresco et al., 2015). For example, it was proved that levels of the stilbenes varied in different portions and seasons of the varieties of mulberries (Zhou et al., 2013). The decrease of resveratrol production during maturation was reported by Jeandet et al. (1991). They showed that the

highest resveratrol produces in July (A month before maturity) and the lowest in September.

The dispersion of wild grape and peanut abundant production in Guilan it should be considered that the waste portions of these plants could be used as an excellent source of natural antioxidants in nutraceutical, food and medicinal industries.

Conclusions

Plants used in the present study, especially berries, are a rich source of phytochemicals. Hence, extensive researches in northern Iran are necessary for isolation and characterization of the bioactive compounds. Among these, wild grape and peanut are the best sources of the resveratrol, which could be isolated and purified. This needs to be more reserched.

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