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A comparative study of phenolic, anthocyanin, flavonoid compounds and antioxidant properties of medicinal fruit of *Vaccinium arctostaphylos* L. (case study: three regions of Talesh, Iran)

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Abstract

Plants are rich sources of phenolic compounds (flavonoids, tannins and anthocyanins) that are considered as the most important natural antioxidants. *Vaccinium arctostaphylos* is a shrub that belongs to Ericaceae family and has long been used in Iran traditional medicine due to its effects such as reducing blood glucose and pressure. The main metabolites of *V. arctostaphylos* are phenols and particularly anthocyanins that have strong antioxidant properties. A comparative study of phenolic, anthocyanin and flavonoid compounds and antioxidant properties of methanol extracts of *V. arctostaphylos* fruits that are collected from highlands of Talesh (Gilan Province) was done as a replacement for synthetic antioxidants. Prepared extract using acidic methanol and phenolic compounds and antioxidant properties were measured by UV-Vis spectrophotometer. Studying methanol extracts of different regions revealed that the maximum amount of phenolic compounds in extracted powder and anthocyanins and flavonoid in fresh fruits in Chubar region was higher compared to other regions. The results showed this species is a rich source of phenolic compounds and can be used in food and pharmaceutical industry as an antioxidant compounds.

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Introduction

Natural products are rich sources of bio-active compounds and a plant hierarchy can be placed in this category as a source of a wide range of antioxidant compounds (Mishra *et al.,* 2008). Most herbs and herbal infusions have often been used in traditional medicine and have antioxidant and drug properties due to the presence of phenolic compounds, especially flavonoids (David & Vikza *et al.,* 2006). Plants compromise apparently with their environment and nature in terms of adaptation and this adaptation is associated to environmental factors and plants react consciously to environmental stimuli that bio-climatic and soil conditions can be considered more important among other factors. Therefore, all these factors are more or less involved in plant's active ingredients (Mahdavi Meymand and Mirtajaldini, 2006). Gilan province in northern Iran is among rainy regions of state and its varied topography of coastal plain and plain-mountain conditions caused rich vegetation. There are about 1,000 plant species according to botanical resources of Iran in Gilan, that nearly half of these plants are medicinal species with various usages (Eslah, 1996).

Anthocyanins are flavonoid pigments and are responsible for red, purple and blue colors of fruits and flowers (Buchert *et al.*, 2005; Jangmin *et al.*, 2005). As Anthocyanins have anti-cancer, anti-oxidant, anti-angiogenic and anti-inflammatory properties, help the body health (Kong *et al.*, 2003; Rusi *et al.*, 2003).Kianbakht *et al.* (2013) improved lipid profile in hyperlipidemic patients taking the fruit. Phenolic compounds include a large group of secondary metabolites; have several ecological and physiological roles such as antioxidant and defense (Andrei *et al.*, 2009).Ayaz *et al.* (2005) extracted high amount of phenoloc acids in this species. Flavonoids and other phenolic compounds are wide spread among plants and diverse biological activity of these compounds has been reported in many of studies such as anti-oxidant, anti-microbial, anti-inflammatory and vasodilator (Jamshidi *et al.*, 2010). Antioxidants are important compounds that protect body against damages resulted from oxidative stresses by free radicals. There are different types of radical scavenors in body that most of them are provided from fruits, vegetables and beverages (Halyol, 2000; Maxwell, 1995).

Vaccinium arctostaphylos L. is a shrub belong to Ericaceae family (Sedaghathoor *et al.*, 2005) and *V. arctostaphylos* fruit is rich in anthocyanins and cause short-term treatment of cholesterol (Kianbakht *et al.*, 2013). Investigating its metabolites shows that ripe fruits contain three main anthocyanins and introduces this plant as an important medicinal plant (Nick Avar *et al.*, 2003). It has properties such as anti-diarrheal, free radical scavenger, protector of cells against chemicals damage including environmental pesticides, decreasing lipid peroxidation and protecting liver against various stresses that is due to high levels of antioxidant substances in this plant (Milbary *et al.*, 2007; Mirheydar, 1996). In fact, detection and investigation of active components of this plant is necessary according to therapeutic effects and medicinal properties. As the plant compounds of different weather conditions are diverse, it is necessary to examine compounds in numerous experiments in different regions. The extraction of chemical compounds have investigated for the first time in Iran and this study aimed to identify indigenous population of *V. arctostaphylos* from three different regions of Talesh, compare their phytochemical characteristics and identify the best ecotype in terms of the highest amount of chemical compounds and domesticate and crop out it so that it is used in food industry, supplying drug and export.

Materials and methods

Materials

The fruits used in this study were collected randomly in August 2013 from Talesh city, high lands of Asalem, Khotbesara and Chubar areas (west Gilan Province) (Tables 1 and 2) and were frozen until testing at -18°.

Extraction

Fruits were dried in oven at 40°C, then where grinded in a mortar and pestle and phenolic extract was prepared by soaking in 80% methanol solvent. Thus, 100 ml solvent was added to 10 g of fruit powder and the mixture was stirred for 24 h at 50°C in a shaking incubator. After this step, the solid part was separated by an ordinary filter paper and the extract was concentrated by rotary evaporator under vacuum at a temperature of 40°C and finally, it was converted into powder by freeze-dryer. The Powder was placed in an air-tight container until use at -18°C in freezer (Arab Shahi and Yuruj, 2007). All materials that were used in this study were prepared with high purity from Merck Company.

Measuring the total amount of phenolic compounds

Phenolic compounds are important constituents of plants because of antioxidant properties that have important role in removing free radicals and preventing hydrogen peroxide conversion into free radicals (Jimuh *et al.*, 2008; Nik *et al.*, 2003). One method to evaluate the antioxidant effects of plants is to use free radicals and removing this radical (Yu *et al.*, 2002). The total phenolic content was measured by Folin-Ciocalteau method (S Linkard and Singleton, 1997) based on standard curve equation for methanol extract and dry weight of plant and results were obtained in mg tannic acid per gram of extract powder.

(y= 0.0354x + 0.2583, R2=0.9822).

Different amounts of extract containing tannins (0.02, 0.05, 0.1 ml) were poured into test tubes and its volume was reached with distilled water to 0.5 ml. Then, 0.250 ml Folin- Ciocalteau reagent and later 1.25 ml of sodium bicarbonate solution was added. Tubes were vortexed and then left for 40 minutes at ambient laboratory and then their absorption was read using spectrometer at a wavelength of 725 nm. Total phenolic was calculated as tannic acid equivalent from calibration curve and results were expressed in mg tannic acid per gram of extract powder. (y= 0.0354x + 0.2583, R2=0.9822).

The measurement of total flavonoid compounds

Standard solutions preparation: 0.1 g routine was dissolved in 100 ml of 50% methanol in order to prepare 1000ppm routine solution. Solutions of 10, 20, 30, 40 and 50 mg/L were made of 1000 mg/L solution. 1 ml of standard or extract and 1 ml of $AlCl_3.6H_2O$ solution were mixed in order to measure the amount of flavonoid. After 40 minutes, solution absorbance was read in the presence of control at 415 nm wavelength (Ziyukovik *et al.*, 2009).

Evaluation of free radical scavenging rate

Evaluating antioxidant capacity can be done in two enzymatic and non-enzymatic ways. Neutralizing feature of radical DPPH (2,2-diphenyl-1 picryl-hydrazyl) in order to evaluate the non-enzymatic antioxidant capacity (Shimada *et al.*, 1992). DPPH is a purple compound that obtaining electron becomes yellow. For this purpose, 1 gram of fruit tissue was kept in a tube with 10 cc of 85% methanol for one hour at room temperature until extraction to be done. After extraction, the extract was filtered through filter paper and was centrifuged at 3000 rpm for 5 min. 0.5 ml of fruit extract with 2.5 ml of DPPH (mM 1/0) was kept in dark place at room temperature for 30 min and then the absorbance of samples were read at 517 nm wavelength. DPPH scavenging percentage was calculated by the following equation:

[(A control – A sample)/A control]×100 =I%.

In this equation, A sample and A control suggest optical absorbance of negative control (no extract) and extract, respectively and I% shows DPPH free radical scavenging.

Determining anthocyanin compounds

Anthocyanin Measurement

5.0 g of each sample (fruit), 0.5 cc of hydrochloric acid, 90 cc of n-propanol was transferred into a glass vial containing 90 cc of distilled water, lid was closed and it was heated up in a Water bath with boiling water for 5 minutes, then was kept in a dark place for 3 hours in order to complete the anthocyanin concentration, later, the solution was centrifuged in

order to reach a certain mode of floating on surface. The sample absorbance at 535 nm wavelength was read with a spectrophotometer and the amount of anthocyanin was compared (Mezomdar and Mojumdar, 2003).

Anthocyanin amount was calculated by the following equation:

e×b×c /a×d

Where a= sample weight, b= the amount for measurement and c= total amount, d=b/c (50 cc)

Statistical analysis

All experiments were performed in triplicates. Data obtained from measurements were carried out in three populations and were analyzed by one way variance analysis (ANOVA) using 16SPSS version software. Means were grouped and compared using Duncan's multiple range test at probability levels of $p<0.05$ and $p<0.01$ after determining the significant differences between data.

The cranberry fruit was collected from one of its natural habitats (Talesh highlands, Shagerdkoh, Asalem, located at an altitude of 1600 meters above sea level and at 37°35´N latitude and 48°42´longitude. Based on the recommendations by [16], to extract the seeds, the fruits were first placed in a container with water, and then were completely soaked with being pressed for a short time. After this stage, more water was added so that the pomace is floated in the water, and healthy seeds are deposited. This process may be repeated several times to properly separate the pomace from the seeds. The pomace residue was separated from water and the precipitated seeds. Then, using a suitable method (e.g., the use of a sieve with small holes or using filter paper), the seeds were separated from water and some fine debris; then, they were surface sterilized with 1% sodium hypochlorite and washed several times with distilled water. For breaking the dormancy of seeds, different physical, chemical and pre-chilling methods were used:

Results and discussion

Phenolic, anthocyanin, total flavonoid compounds and antioxidant properties of fruits collected from three regions of Talesh were analyzed and results showed that the highest antioxidant activity is related to Chubar region and the lowest activity is related to Khotbesara region (Fig. 1). But there is no significant difference between Chubar and Asalem regions. The high antioxidant activity can be attributed to phenol and anthocyanin amount, as there is a good correlation between polyphenols and anthocyanins and antioxidant activity. The antioxidant activity of fruit depends on harvest time, growth, cultivar and environmental conditions. It has been reported that phenol amount and antioxidant activity are significantly influenced by race and growing season. The amount of phenol and anthocyanin are more influenced by race than growing season, while the antioxidant activity is more influenced by growing season (Chen *et al.*, 2006). The difference between qualitative and quantitative features of cultivars, is affected by environmental conditions (temperature, light and agricultural activities) apart from the difference in race. We can add these features in cultivars providing proper conditions and use them for human health. In all plants, there is direct relationship between antioxidant activity and phenolic and flavonoid compounds.

Table 1. Geographic position of 3 areas in Gilan.

Areas	«Latitude	ASL
Asalem	37˚ 39´N، 48˚ 55´E	1600
Khotbesara	38˚ 02´N، 48˚ 46´E	940
Chobar	38˚ 09´N، 48˚ 41´E	2094

Peppermint extract has high phenolic and flavonoid compounds and indicates high antioxidant activity (S. Witt *et al.*, 2007). Rosemary extract has been proven to have high antioxidant activity and there is direct

relationship between this activity and phenolic content of plant (Almasta *et al.*, 2006). This relationship has been shown by Coca and Karadeniz (2009) in blueberries that grow around the Black Sea and Fang *et al.*, (2009) in *Myrica rubra* reported direct relationship between amounts of total phenolic, anthocyanin and flavonoid compounds. Fig. 2 shows the highest amount of phenol is related to Chubar region and the lowest amount is related to Khotbesara region and there is statistically significant difference between Chubar phenol and two other region at significance level of p>0.01. However, according to the results we can say that the amount of phenolic compounds is mainly dependent on the difference between cultivars and species and these results are obtained by Yang *et al.*, (2009).

Table 2. Soil properties in 3 different areas in Gilan.

Areas	Soil Depth (cm)	Soil texture	EC(ds/m)1	pH
Asalem	0-30	Loam	1.23	5.61
Chobar	0-30	Loam-Sandy	1.22	6.28
Khotbesara	0-30	Loam-Sandy	1.61	5.66

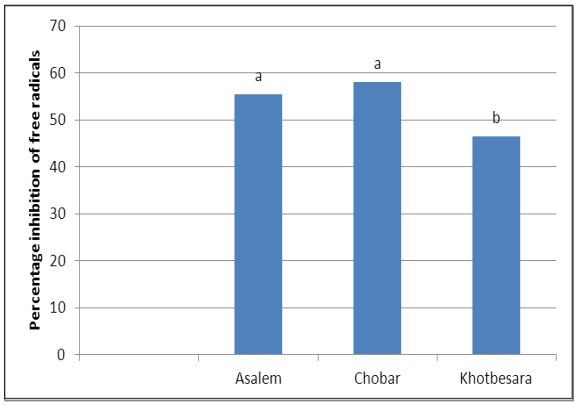

Fig. 1. The amount of Antioxidant in *Vaccinium arctostaphylos* fruit in three different areas in Gilan.

It has been reported that distribution and amount of phenolic compounds and antioxidant activity are affected by degrees of maturity, cultivar, agricultural operations, latitude, growing season, storage conditions, processing and growth regulators (Kadir *et al.*, 2009). Rising sun light, temperature and plant lifetime increase accumulation of phenolic substances (Hernandez-Jimenez *et al.*, 2009) and increase amount of phenol production in plants increasing the activity of enzymes especially Phenyl alanine ammonia-lyaseT PAL that plays important role in the conversion of phenylalanine (Shikimic acid production cycle) to coumaric acid precursor that in turn is prerequisite of molecules involved in production of phenolic compounds in plant (Valamoti *et al.*, 2007). In studies conducted by researchers it is identified that there is direct relationship between the amount of antioxidant effect and phenolic compounds (Kaavr and Kapoor, 2002, George *et al.*, 2005). Phenols and polyphenol compounds such as flavonoids are widely found in food products and it has been shown that they have significant antioxidant activity (Van Acker *et al.*, 1996). Studies have shown that increased levels of flavonoids in diet can lead to a reduction of some diseases in human (Hertog *et al.*, 1993).

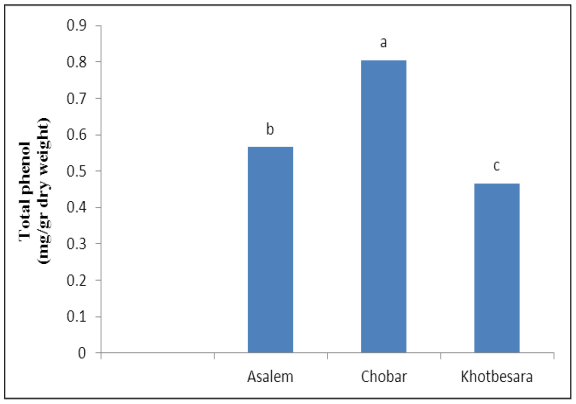

Fig. 2. The amount of total Phenol in *Vaccinium arctostaphylos* fruit in three different areas in Gilan.

Flavonoid content was obtained based on standard curve equation for the same set in mg of routines per gram powder. In Fig. 3 it can be seen that Chubar has the highest amount of flavonoid compared to another regions and there is significant difference at level of p>0.01. The lowest amount of flavonoid belongs to Khotbesara region. Anthocyanin amount will be different due to growth environmental conditions,

race and harvest time. This is similar to results obtained by Huliya (2007), who reported that anthocyanin level varies depending on environmental conditions, fruit size and cultivar genotype. Fig. 4 shows that the maximum amount of anthocyanin is related to Chubar region and Kotbesara has the minimum amount of anthocyanin. It is observed that there is no significant difference between Asalem and Khotbesara. In total, according to data from this study we can introduce Chubar as the richest sample in terms of phenolic compounds and antioxidant activity compared to Asalem and Kotbesara regions. *V. arctostaphylos* fruit that is one of the native fruits of Talesh, has high antioxidant potential due to the large amounts of phenolic compounds and hence can be a rich source of natural antioxidants in producing various products such as jam, marmalade, chutney and other food and drug products where oxidation likely occurs as the presence of lipid.

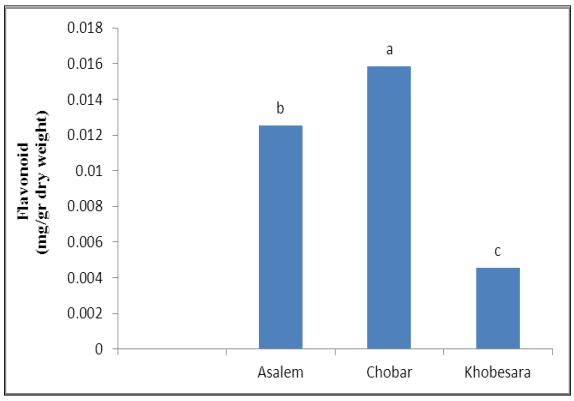

Fig. 3. The amount of Flavonoid in *Vaccinium arctostaphylos* fruit in three different areas in Gilan.

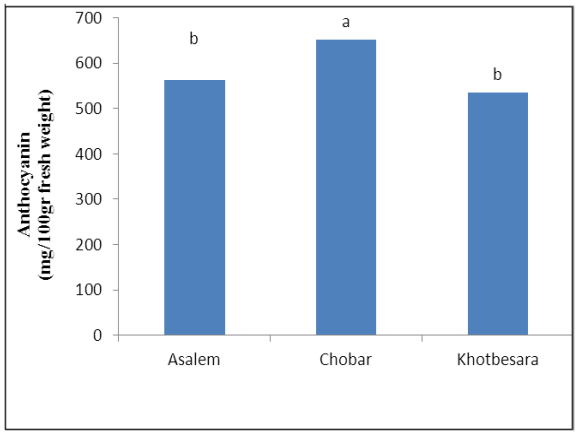

Fig. 4. The amount of Anthocyanin in *Vaccinium arctostaphylos* fruit in three different areas in Gilan.

Based on the results of this study, in terms of antioxidant effect and total Phenolic and flavonoid compounds, it can concluded this plant is potentially used in treatment and prevention of many diseases such as diabetes, inflammation, cancer, liver damage, Alzheimer and heart coronary artery disease. Since antioxidants are used in food and drug industry as protective material and this plant contains phenolic and flavonoid compounds, and this compound is responsible for antioxidant effects and recently flavonoids are used as anti-virus, anti-rheumatic and preventive of cardiovascular diseases, therefore, use of such plants can be raised in food industry and reducing the progression of disease in a wide variety of diseases.

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