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Original Article

Ecological Requirement, Ethnopharmacology, Phytochemical and Antioxidant Capacity of *Vaccinium arctostaphylos* L. in Gilan Province (North of Iran)

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Abstract

This study is investigate to ecological characters, ethnopharmacology, total phenols (TP), flavonoids (TF), anthocyanins (TA) contentand antioxidant capacity in leaves and fruit of *Vaccinium arctostaphylos* L. from two natural habitats from Gilan provinve, which has been used as a heart tonic ,anti diabeteand anti tumor. Ecological requirements were obtained in many field observation, ethno pharmacological survey was carried out among the well known indigenous herbal practitioners (60-78 ages) in South west of Gilan. The leaves and fruits of *Vaccinium arctostaphylos* L. were collected from two natural habitats (Khotbe sara,1600 m and Asalem, 1300 m) during Sep to Oct 2013, respectively. Methanol extracts were obtained by maceration, TP, TF and TA were determined by spectro photometrically, antioxidant capacity were measured by total antioxidant capacity (TAC), reducing power (RP) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) in compare of BHT and BHA antioxidant standard and Quersetin content was messured by HPLC method. Results were showed that *Vaccinium arctostaphylos* L. is usually wild grow in silty loam soils (1300-1600m) and its fruit was appeared from June to September .In both regions phenolic components had significant variation, ranging from TP content(263.17-471.26 mg GAEg-1),TF(186.18-289.17 mg QUE g-1) , TA (74.3- 145.29 mg CydEg-1) and quersetin content (136.13-218.2 ppm respectively in 1300-1600m. Generally the fruits extract especially in 1600m had more source of phenolic componds, so was showed better antioxidant capacity (IC50 value 2.98±0.51 µg/ml) to will be confirmed the traditional uses of plant in these regions as an good anti-inflammatory and anti diabetic.

Keywords: Antioxidant, Autecology, Ethnopharmacology,TP, TF, TA, Gilan, *Vaccinium arctostaphylos* L.

Introduction

Edible berries are rich in antioxidants, anthocyanins and other phenolic compounds (TF, TP and TA) have demonstrated the ability to reduce risk for development of cancers, cardiovascular disorders, diabetes, aging-diseases and urinary tract infections (UTI), so berries and leaves of *Vaccinium* L. (Ericaceae family) have been an important source of natural antioxidant potential [1].

Invitro studies showed that the Fruit, leaf and even stem extracts of *V. mytillus* and *V. bracteatum* Thunb. will be effective as anti-diabetic, antioxidant and antimicrobial [2] and could be used to treatment of periodontal, infection against hepatitis C and inhibit human leukemia and breast, colon, lung and prostate cancer cells invitro [3-7].

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The fruits blueberry species have many pharmaceutical activity due to they are very good source of fiber, vitamin K, C, copper, manganese and contain significant amounts of anthocyanadins, antioxidant compounds that anthocyanins are: malvidins, delphinidins, pelargonidins, cyanidins, and peonidins. In addition to their anthocyanins, blueberrries also contain hydroxycinnamic acids (caffeic, ferulic, and coumaric acid), hydroxybenzoic acids (gallic and procatechuic acid), and flavonols (kaempferol, quercetin and myricetin). Blueberries also contain the unique, phenol-like antioxidants pterostilbene and resveratrol [8,9]. As well as in recent years, a high scavenging activity of berry extracts has been shown in several studies and showed the phenolic compounds in berry extracts inhibited human low-density lipoprotein (LDL) and liposome oxidation may significantly reduce the mortality rates of cancer, cardiovascular disorders, diabetes and other degenerative diseases caused by oxidative stress [10]. Furthermore, antioxidant capacity may be correlated with total phenolic and anthocyanin contents in blueberries [1,11]. So The aim of this research was to determine changes inanthocyanin, polyphenol (TP, TF) contents and antioxidant activity for *Vaccinium arctostaphylos* from two natural habitats in Gilan province, North of Iran.

Material and Methods

Ecological requirements and ethno pharmacology

In many field observation, ecological requirements and traditional pharmaceutical knowledge about *Vaccinium arctostaphylos* L. in two natural habitats which are isolated in mountainous regions (Khotbeh sara, 1600m and Asalem, 1300m) with in latitudes of 55° 57' 55" to 52° 57' 55" and longitudes of 25° 46' 37" to 15° 42' 37", respectively in South west of Gilan province , respectively. In this study we selected two elderly rural practitioners (especially bonesetters-65 y) and the traditional data have been interviewed about local name of plants, part used and its medicine effect to treat of their current diseases, then all obtained data from questionnaires were compared with the findings in vivo and invitro experiments in other similar reports.

Plant Material

In both region , the leaves and fruits of plant were collected in July- september. 2013 . plant voucher specimen was identified in RCMP (Research center of Medicine Plant) of Islamic Azad University of Gorgan branch and was preserved on (No. HRCMP:489).

Extract Preparation for Phytochemical and Antioxidant Tests

One gram of plant parts with 100 ml (methanol 80%) were extracted by maceration. Extracts were filtered with Whatman No. 1 filter paper. The filtrates obtained from extracts were evaporated into dry rotary evaporator at 40 °C and were stored at 4 °C [12].

Chemicals

2,2'-diphenyl-1-picrylhydrazyl (DPPH) and quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one) were purchased from Sigma Chemical Co. (St., Louis, USA). Gallic acid, Folin-Ciocalteu reagent , BHT (butylated hydroxytoloane), BHA (butylated hydroxyanisole) and methanol were purchased from Merck Co. (Germany).

Antioxidant Activity Tests

Reducing Power assay

This assay is based on Arabshahi-Delouee method. First, The dried extract (12.5–1000 µg) in 1 ml of the corresponding solvent was combined with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (K3Fe(CN)6; 10 g l-1), after the mixture was incubated at 50 °C for 30 min. Then, 2.5 ml of trichloroacetic acid (100 g l-1) were added and the mixture centrifuged at 1650g for 10 min. Then, 2.5 ml of the super natant solution was mixed with 2.5 ml of distilled water and 0.5 ml of FeCl3 (1 g l-1), and the samples absorbance was measured at 700 nm [13].

1,1-diphenyl-2-picryl hydrazyl radical scavenging capacity Assay

The ability of the extracts for free radical scavenging was assessed by the method suggested by [13]. Briefly, 1ml of a 1mM methanolic solution of DPPH was mixed with 3ml of extract solution in methanol (containing 12.5–1000 µg of dried extract). The mixture was then vortexes vigorously and left for 30 min at room temperature in the dark. The absorbance was measured at 517 nm and activity was expressed as percentage DPPH scavenging relative to control using the following equation:

$$\text{DPPH scavenging activity (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Total Antioxidant Capacity

This experimental procedure was adapted from Arabshahi-Delouee method, which is based on the reduction of Mo (VI) to Mo (V) by the sample and observation of a green phosphate/Mo (V) complex at acidic pH. An aliquot of 0.1 ml of sample solution, containing 12.5-1000µg of dried extract in corresponding solvent, was combined in a tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). They were incubated in a thermal block at 95 °C for 90 min. Then we got cold the samples and measured their absorbance at 695 nm. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent was used for the sample, and was incubated under the same conditions as the rest of the samples [13].

Determination of Total Phenolic Content

It was determined using the Folin-Ciocalteu Reagent. Total phenolic content was estimated by the Folin Ciocalteu method, based on the procedure suggested by Pourmorad *et al*. [12] .Then 0.5 ml of plant extracts or gallic acid (standard phenolic compound) was mixed with Folin Ciocalteu Reagent (5 ml) and aqueous Na2CO3 (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenols were determined by colorimetry at 765 nm. Gallic acid was used as a standard for calibration curve. Total phenol values were expressed in terms of mg equal gallic acid in 1 gr powder dry plant [12].

Determination of Total Flavonoid Content

Total flavonoids content were determined by aluminum chloride method. Extract plants (0.5 ml) were separately mixed with 1.5 ml of solvent, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. They were kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with a spectrophotometer. Total flavonoid values were expressed in terms of mg equal quercetin in one gram powder dry plant [12].

Total Anthocyanin Determination

Total anthocyanin content was determined by the pH-differential method described by Giusti [14], by using 2 buffer systems: potassium chloride buffer, pH 1 (1.86 g KCl in 1 L of distilled water, pH value adjusted to 1.0 with concentrated HCl) , and sodium acetate buffer, pH 4.5 (54.43 g CH3CO2Na·3H2O in 1 L of distilled water, pH value adjusted to 4.5 with concentrated HCl). The sample diluted with corresponding buffer and the absorbance was measured at 510 and 700 nm. Total anthocyanins were calculated as cyanidin-3-glucoside according to the following equation:

$$TAC = \left(\frac{A \times MW \times DF \times 100}{MA}\right)$$

A= (A510 −A700) pH1−(A510 −A700) pH4.5
MW: 449.2 g/mol for cyanidin-3-glucoside
DF = dilution factor; MA: 26900

HPLC analyzis

Statistical analysis
For all assays, data were expressed as means±S.E. and differences at $P<0.05$ were considered statistically significant.

Results

The content of total phenols (TP), total flavonoids (TF) and total anthocyanin (TA) in leaves and fruits in two *Vaccinium arctostaphylos* (1300-1600 m) which are given in Table 1. TP content in Asalem region (1300m), was determined with Folin-Ciocalteu assay ranged from 268.17±8.12 to 382.15±12.17mg of gallic acid equivalents (GAE) per g in leaves and fruit respectivelyin , as well as TF content ranged from 186.18±18.34 in leave to 253.75±28.1 17 mg of Quercetin equivalents (QUE) per g in fruit and TA content ranged from 74.17±22.13 mg of Cyanidin equivalents (CYE) per g In leaves to 105.12±13.23 mg CydE/g in fruit. the same species in Asalem region (1300m), TP, TF and TA content were higher and ranged from 338.14±1.81 mg of GAE per g, 218.17±62.13mgQUEg- and 82.98±5.16 mg CydE/g in leaves, respectively and 471.76±2.13 mgGAEg-1, 289.17±16.47 mgQUEg- and 145.29±9.31 mg CydGE/g in fruits, respectively in Khotbe sara (1600m).On the other hand in both regions, fruit extract had higher content of TP,TF and TA in Table 1, Fig 1-2, and leave and fruit extracts in 1600m with IC50=3.2±0.13 µg/ml in DPPH method, respectively had higher content of IC50 to free radical scavenging to compare of BHT and BHA standard (Table 2) and high content of quercetin (218.02 µg/mg) was found in plant fruit extract in 1600 m (Fig. 4, 5).

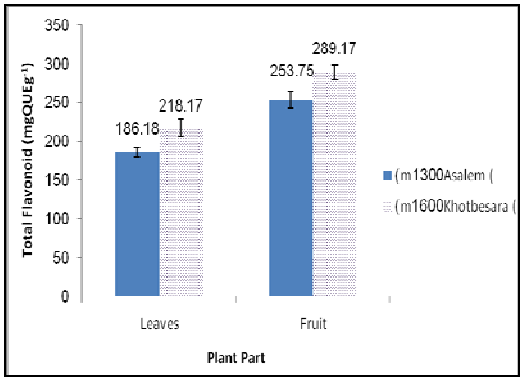

Fig. 1 Total flavonoids content (mg QUE/g) in different parts of *V. arctostaphylos* L. in Gilan province

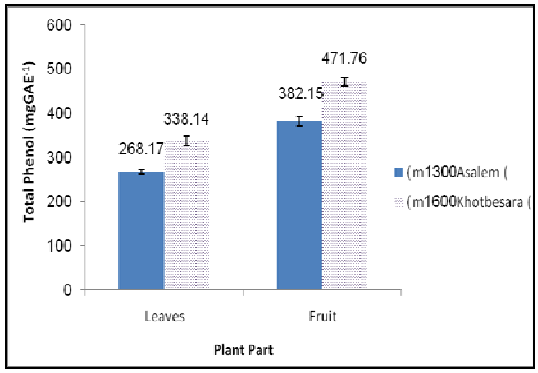

Fig. 2 Total phenols content (mg GAE/G) in different parts of *V. arctostaphylos* L. in Gilan province

According to Table 2 and Fig. 2,3,4 maximum antioxidant activity in DPPH, RP and TAC methods was observed from fruit extracts, especially in1600m and DPPH method with IC50=3.2±0.13 µg/ml, in so much, the fruit extract have more TP TF, TA and quercetin content than leave extract in two regions.

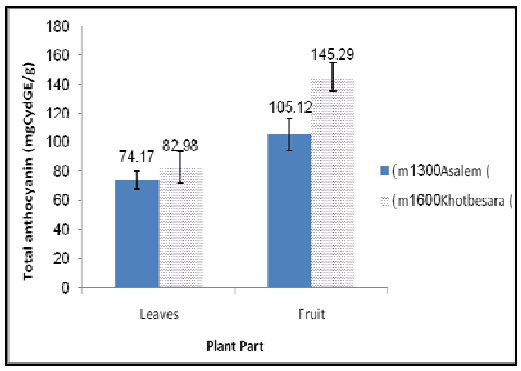

Fig. 3 Total anthocyanin content (mgCYDE/g) in different parts of *V. arctostaphylos* L. in Gilan province

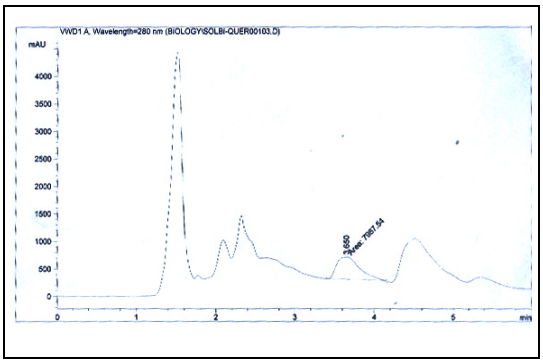

Fig. 4 The quercetin content of the fruit extract of plant in Khotbesara region(1600m)

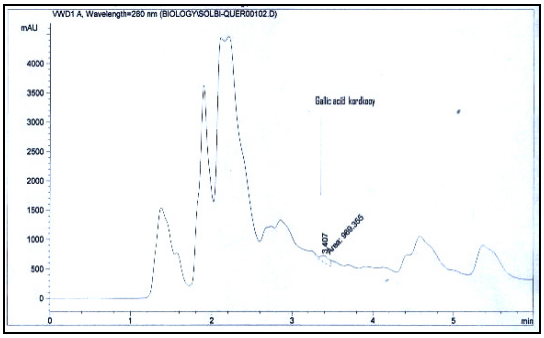

Fig. 5 The quercetin content of the fruit extract of plant in Asalem region (1300m)

Table 1 Total Phenols (TPh), Flavonoids (TF) and Anthocyanins (TA) in Gilan province (1300-1600m)

Metabolite	Total Flavonoid (mg QUEg^{-1})		Total Phenol (mg GAEg^{-1})		Total anthocyanin (mg CydGE/g)	
Parts	Asalem (1300m)	Khotbesara (1600m)	Asalem (1300m)	Khotbesara (1600m)	Asalem (1300m)	Khotbesara (1600m)
Leaves	186.18±18.34	218.17±62.13	268.17±8.12	338.14±1.81	74.17±22.13	82.98±5.16
Fruit	253.75±28.1	289.17±16.47	382.15±12.17	471.76±2.13	105.12±13.23	145.29±9.31

Values are an average of 3 replications ± SD

Table 2 Antioxidant activity of *V. arctostaphylos* L. in Gilan province 1300-1600m

Part Antioxidant	Khotbesara (1600m)		Asalem (1300m)		BHA	BHT
Activity IC50 (µg/ml)	Leave	Fruit	Leave	Fruit		
TAC	9.11±0.88	7.09±1.08	14.06±0.16	10.01±0.16		
RP	8.26±0.81	5.83±0.90	22.04±1.14	11.16±0.68	3.85±0.351	3.13±0.404
DPPH	3.20±0.13	2.98±0.51	5.20±0.51	4.80±0.32		

Ethno pharmacological survey showed that dry leaves and especially the fruits of *V. arctostaphlos* L. with localy name '*Gharehghat and Siahgileh*' is one of the most wild edible fruit ,which have been used in traditional medicines of rural Gilan province as a heart tonic , anti inflammation, astringents, diuretic , anti virus, and anti ulcer drugto treat of diabetes, hypertension,diarrhea, arrhythmia, edema, cataracts, hyper LDL, leg cramp, atheriosclerosis, amenorrhea, dysmenorrheal, UTI, waris, arthritis rheumatoid, and breast tumor with combination with other herbs such as below:

For treat of diabetes: consumption of 20 - 30 fruit per day or the mixed of (1 g of *Nigella sativa* + 2 g of *Urtica dioica* plus a little of *Pistachia lentiscus* and oliveleaves) after per meal.

Ulcer: consumption the Dusin per day (the mixed of *Nigella sativa* in honey)in plus of little of *Ferula assa foetida* gum.

Hypertension, arrhythmia and atherosclerosis: infusion of 5- 10 fruit for 10 mimute with 1 tea spoon of Dusin , *Foeniculum vulgare* and *Urtica dioica*

Discussion

According to Table 1,2, results in present study were suggested that the TP, TF and TA compounds contributed significantly to antioxidant capacity of *V. arctostaphylos* L. against free radical, especially in fruit extract and with DPPH test in in 1600m (Khotbesara), due to their traditional uses as anti inflammation, anti diabetes and hypertension.

As maximum antioxidant activity in DPPH, RP and TAC methods was observed from fruit extracts, especially in 1600m and DPPH with IC50=3.2±0.13 µg/ml, because in so much the fruit extract have more TP TF, TA and quercetin content than leave extract in two regions. So comparing the results of our research with another results, the fraction of TP , TA content and anti oxidant activity in diferent cultivar of *Vaccinium* species were varied ranges and it was in agreement with the findings of Dragovi and Zheng [11,15] , who found richness in phenolic contents (313 mg-528.2 mg of GAE per 100 g of TPC) in the same blueberry cultivar [15]. Similar to our results in Table 1 and 2, other authors showed that there are High correlation ($p<0.05$) was detected between the mean anti oxidant activity especially specially in DPPH method to the TP , TF and TA content in plant extract, especially in Khotbe sara region, 1600m in Fig. 1,2,3.

These results were in agreement to many researches, who reported that , there is direct relationships between TP content and their antioxidant activity in variety of species (*Sylibum marrianum, Heracleum gorganicum, Artemisia anuua, Onosma dichloraanthum* and *Heracleum gorganicum* [17,18].

In confirming to our ethnopharmacology results, Grace showed that treatment by gavage (300-500 mg/kg) of *V. myrthilus* extract with rich phenolic content (287.0±9.7 mg/g) and anthocyanin contained (595±20.0 mg/g (cyanidin-3-glucoside equivalents), which could lowered elevated blood glucose levels by 33 and 51%, respectively to comparable of the known anti-diabetic drug metformin[19]. More over another study showed that the leaf extract of *Vaccinium angustifolium* contained high concentrations of chlorogenic acid (100 µg/mg extract) and a variety of quercetin glycosides that were also detected in the fruit and stem extracts. Flavan-3-ol monomers (+)-catechin and (−) -epicatechin were found in plant fruit and have good effect in anti oxidant and hypoglycemic activities [20].

Quercetin and quercetin 3-O-glycosides are responsible to prevention and treatment of insulin resistance and other metabolic diseases and the antidiabetic activity of V. vitis crude berry extract mediated by AMPK(insulin-independent AMP-activated protein kinase in AMPK pathway [21].

To confirming of our ethnopharmacological survey, in a many previous study, phenolic compounds from blueberry extracts inhibited human low-density lipoprotein (LDL) and liposome oxidation , Thus, high fruit consumption may significantly reduce the incidence and mortality rates of cancer, cardiovascular disorders anddiabetes ,so furthermore antioxidant capacity may be correlated with total phenolic and anthocyanin contents in blueberries and did change significantly during fruit rippening , as total phenolics contents increased during ripening with maturity [1,11,15,22].

However, evidence has shown the relationship between a diet rich in fruit and leaves of Whortle berries (*V.arctostaphylos* L.) are rich antioxidant in sources of polyphenols (flavonols, phenolic acids, anthocyanins , quercetin and ellagitannins) , vitamins C and E, which found to decrease of cardiovascular disease and certain types of cancer,

degenerative diseases and increased the resistance to oxidatively induced DNA damage[23-26]. These data demonstrated that leaf and fruit extract of *V.arctostaphylos* L. which growing in mountain area possess better antioxidant activities against free radical produced could be used as an alternative therapy for anti diabete and help prevent various free radical related diseases [26,27] .

More attention has been focused on the protective biochemical function of naturally occurring antioxidant in biological systems and on the mechanism of their action. Poly phenols are the most secondary antioxidant compounds in medicinal plants which have important role in blocked activity of free radicals and so there was a positive correlation between total phenolic content and antioxidant activity.

Conclusion

The results confirmed that the *V. arctostaphylos* L. which wild grown in North of Iran as a source of phenolic compounds with high antioxidant activity and there are different levels of TP, TF and TA compounds and antioxidant activity according to the plantparts and their natural habitats. Overall, the results of this study show the great potential of Whortle berries (*V. arctostaphylos* L.) in Gilan province for the development of foods rich in compounds with antioxidant properties.

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