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Original Article

Anthocyanin Isolation from *Berberis integerrima* Bunge Fruits and Determination of their Antioxidant Activity

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ABSTRACT

Background: Anthocyanins are famous members of flavonoid group with multifaceted effect including anti-inflammatory, anti-oedema activities, antioxidant and antitumor activity. *Berberis integerrima* is candidate in treatment of gastrointestinal diseases, bleeding, fever, malaria and hepatitis. The aim of this study is to isolate anthocyanin of *Berberis integerrima* Bunge fruits (AFBI) and measurement of free-radical-scavenging activities by different methods. **Materials and Methods:** AFBI isolated by column chromatography. Anthocyanin content was measured by PH-differential method and antioxidant activities of AFBI quantified by various methods (DPPH, FRAP, ABTS) as well as lipid peroxidation. **Results**: Anthocyanin content is 14.36 ± 0.33 mg/g before purification by column and 34.51 ± 0.42 mg/g in AFBI. IC_{50} of anthocyanin fraction before loading on column are 471.06 ± 1.8 µg/ml, 65.98 ± 0.66 µg/ml and 495.5 ± 1 µg/ml for DPPH, FRAP and ABTS assay, respectively. These IC_{50} after loading on column are 66 ± 0.04 µg/ml, 11 ± 0.16 and 60 ± 2.1 µg/ml for DPPH, FRAP and ABTS assay, respectively. Nitric oxide scavenging values are 53 ± 0.5% and 97.04 ± 0.69% before and after using column for anthocyanin purification. The percentages of lipid peroxidation inhibition of AFBI are about 39% in first day and 95% after 10 days. **Conclusion:** According to results, mentioned method is efficient for anthocyanin purification and antioxidant activity increased significantly after loading sample on this column. *Berberis integerrima* fruits can be noticed as proper source of anthocyanin which able to scavenge different free radicals and protective agent against lipid peroxidation.

Key words: Anthocyanin, *Berberis integerrima* Bunge, ABTS assay, DPPH scavenging, FRAP assay, Lipid peroxidation.

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INTRODUCTION

Anthocyanin are known as large group of red-blue natural pigments giving different color in plants and their products.[1] They belong to flavonoids, a large group of phenolic compounds, divided to aglycons and glycosylated form.[1] More than 400 anthocyanins have been recognized in nature. Anthocyanin play different role in plants such as antioxidants, phytoalexins or as antibacterial agents.[2] Nowadays anthocyanin consider as health benefit compounds.[3] They shows different pharmacological properties comprise improving cognitive decline and neural dysfunction[4] reducing capillary permeability and fragility, inhibitory effect on NO· production and effective in inflammatory diseases which are associated with NO·.[2]

Evidence of many researchers suggests that anthocyanin poses confirmed potential potency in cancer prevention and decrease risk of human breast cancer, human melanoma cancer and human ovarian cancer.[1] Anti-cancer properties of these compounds related to several mechanisms, they are powerful free radial scavenger and have antioxidant effects because of phenolic structure. They are anti-proliferator agents towards several cancer cell lines and can be induced apoptosis. Moreover, they able to inhibit angiogenesis and invasiveness in cancer cells.[5]

Berberis sp. Fruits are rich sources of many bioactive compounds such as anthocyanin.[6] *Berberis integerrima* belongs to Berberidaceae. Synthesis of different secondary metabolites in this plant make it as therapeutic agent in different disease such as gastrointestinal diseases, bleeding, sore throat, fever, malaria and hepatitis.[7]

The aim of this study was to separation of anthoyanin fraction of *Berberis integerrima* Bunge fruits (AFBI), evaluation of anthocyanin content in this fraction and antioxidant activity assessment of this fraction by different methods: DPPH, ABTS radial scavenging assay, inhibition of lipid peroxidation and nitric oxide.

MATERIALS AND METHODS

MATERIALS

All the chemicals used were obtained from Sigma and Aldrich Company (St. Louis, MO, USA).

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Plant material

Berberis integerrima Bunge fruits were manually harvested during August and September 2104, from Kohmar (Fars Province, Iran), and were identified by Ms. S. Khademian in the museum of medicinal plants, Shiraz University of Medical Sciences, Shiraz, Iran (voucher no. P. M. 396).

Extraction

325 g of freeze-dried fruits powder was percolated with ethanol for 48h and the extract concentrated in rotary evaporator under vacuum followed by speed vacuum to get 140 g gummy material.

30 g of the crude extract was then suspended in 0.3% TFA then filtered and shake well with ethyl acetate in decanter. The aqueous phase was collected, this extraction repeated three times. This phase was loaded an amberlite column (2.5×45 cm), then rinsed with distilled water containing 0.3% TFA to remove polysaccharides .The anthocyanin were eluted with 0.3% TFA in methanol then concentrated by rotary evaporator. Yield of extraction and anthocyanin fractionation were 20% and 2.57%, respectively.

Anthocyanin content measurement

The pH-differential method was used to determine the anthocyanin content in the fraction.[8]

0.4 ml of sample was mixed with 3.6 mL of corresponding buffers. Buffers were 0.4 M sodium acetate solution and 0.025 M potassium chloride solution adjusted to pH 4.5 and 1.0 with HCl, respectively. The absorbance of each dilution was measured at 519 nm and distilled water used as blank. Anthocyanin content was calculated by the following equation:

$$\text{Anthocyanin Content (mg/ml)} = (A \times MW \times DF \times 1000) / \varepsilon \times L$$

where A is ((A519 (pH1.0)-A519 (pH4.5)), MW is the molecular weight of anthocyanin (433.2 g/mol), DF is the dilution factor (10), ε is the extinction coefficient (31600 L cm-1mol-1) and L is the path length (1 cm).

DPPH free radical scavenging activity

The antioxidant activities of the fraction were measured by modified method.[9,10]

Fresh DPPH radical solution (100 mM) was mixed with different concentrations anthocyanin fraction. The reaction mixture was incubated at room temperature for 30 min in the dark. The DPPH radical inhibition was measured at 490 nm by using a microplate reader. All measurements were performed in triplicates. The antioxidant activity was calculated as follows:

$$100 - [(A)\ \text{sample} - (A)\ \text{blank}) \times 100 / (A)\ \text{control}].$$

"A": the absorbance

Control: contains DPPH without sample; blank: methanol.

Nitric Oxide radical assay

Nitric oxide (NO) radical scavenging method was performed with slight modification.[9] Sodium nitroprusside in phosphate buffer (0.2 M, pH 7.4) was mixed with each sample and incubated (27 °C for 150 min). After incubation, 100 µL of o Griess reagent was added to each sample and then incubated 5 min at room temperature. Finally, absorbance was measured at 542 nm.

Nitric oxide radical scavenging was determined as follow:

$$[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100 \text{ control sample control}$$

Blank: Absorbance of sample without any reagent

Control: Absorbance of control without sample

ABTS Assay

The base of this assay is the ability of different substances to scavenge 2,2' azinobis (3-ethylbenzthi-azoline-6-acid) ($ABTS^{\bullet+}$) radical. Colorful ABTS radical change into colorless neutral form. These radicals were freshly prepared by adding ammonium persulfate (2.45 Mm) solution to ABTS solution (7 Mm) and kept in dark for 16 h. This $ABTS^{\bullet+}$ solution was diluted with ethanol to gain an absorbance of 0.70 ± 0.02 at 734 nm. 200 µl $ABTS^{\bullet+}$ Solution and 20 µl of tested samples (6.25-3200 µg/mL) and the absorbance was recorded at 734 nm. The antioxidant activity of was calculated by the following equation:

E= (1-(At/Ac)) ×100

where: At and Ac are the absorbance of tested samples and $ABTS^{\bullet+}$ respectively.[11]

FRAP (Ferric-Reducing Antioxidant Power) assay

In the presence of antioxidants, ferric and tripyridyltriazine (TPTZ) reduce and change its color complex. The FRAP reagent included TPTZ solution (10 mM) in HCl (40 mM), $FeCl_3$ (20 mM) and acetate buffer (0.3 M , pH 3.6). The fresh mixture was prepared and incubated at 37 °C.

Then 20 µL of each sample and 180 µL of FRAP reagent were mixed in a 96-well microplate reader then the absorbance of the reaction mixture was measured at 593 nm after incubation at 37 °C for 10 min. Results reported as IC_{50}.[9]

Lipid peroxidation

The thiobarbituric acid (TBA) assay was carried out by the method of Rael *et al.* with slight modifications [9]. Initially mixture prepared in a screw-cap vial contain 4.0 mg of a sample in 4 mL of 99.5% ethanol, 4.1 mL of 2.5% linoleic acid in 99.5% ethanol, 8.0 mL of 0.02 M phosphate buffer (pH 7.0) and 3.9 mL of water then this mixture was placed at 40 °C in the dark. To 0.1 mL of this mixture, 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% ammonium thiocyanate was added. Then 0.1 mL of 0.02 M ferrous chloride in 3.5% hydrochloric acid was added to the reaction mixture, the absorbance was measured at 500 nm. The absorbance was measured at this wavelength every 24 h until the day after that the absorbance of the control reached its maximum value.

Percentage inhibition was calculated by the following expression:

Percentage of inhibition = [(Ao- A1) / Ao] × 100

where Ao is absorbance of control and A1 is absorbance of sample

is absorbance of sample.

Statistical analysis

All experiments were performed three times and results are reported as the mean ± standard deviation.

RESULT AND DISCUSSION

Results of phytochemical studies of *Berberis* species showed that various parts of these plants used for different purpose. *Berberis* sp. fruits have promising nutraceutical compounds with potential health advantages.[12] The former studies demonstrated anthocyanins are effective biological agents in wide range of disease such as cancer, neuralage-related disease.[13]

In this study, anthocyanin of AFBI measurement by the pH-differential method showed that anthocyanin content of fruits before use of amberlite column as a stationary phase is 14.36 ± 0.33 mg/g and anthocyanin content is 34.51 ± 0.42 mg/g after separation of fraction by column.

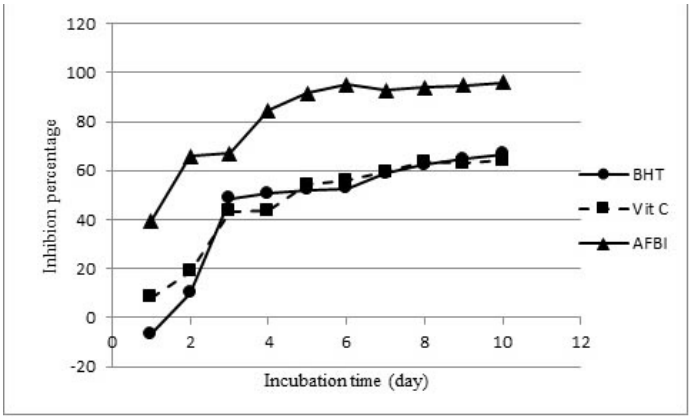

Figure 1: Results of lipid proxidation assay of AFBI and standards.

Table 1: Antioxidant activity of sample before and after loading on column by different methods.

Test / Sample	DPPH IC_{50} (µg/ml)	FRAP IC_{50} (µg/ml)	ABTS IC_{50} (µg/ml)	Nitric oxide % Inhibition
Activity before loading on column	471.06 ± 1.8	65.98± 0.66	495.5 ± 1	53 ± 0.5
Activity before loading on column	66 ± 0.04	11 ± 0.16	60 ± 2.1	97.04 ± 0.69
Quercetin	26.51 ± 0.06	8.69 ± 0.03	25.64 ± 0.02	------

These results showed the effective anthocyanin separation using amberlite. it seems that the value of anthocyanin content of our study is comparable to previous reported including anthocyanin content of orange Nasturtium flowers (*Tropaeolum majus*) was 72 ± 10 mg/100 g,[14] fresh *Rubus glaucus* cv Hull (75 mg/100 g), a hybrid of strawberries (*Fragaria anannassa*) fruit is 71.8 mg/100 g.[15] Anthocyanin content can be affected by seasonal variations.[16] This variation in anthocyanin contents may be due to stability of anthocyanin related to multifactorial such as temperature, pH, presence of phenols and metals complex and structures.[17]

In DPPH assay radical react with hydrogen donor antioxidant and DPPH color convert from purple to yellow. Antioxidant activity related to change of color and measure by reduction of DPPH maximum absorption.[18-19] The IC_{50} of this assay reduce to 66 ± 0.04 µg/mL after loading on column while IC_{50} of Quercetin is 26.51 ± 0.06 µg/mL (Table 1).

FRAP assay engaged to assessment radical scavenging of AFBI. Reaction of TPTZ-Fe (III) complex by antioxidant leads to form TPTZ-Fe (II) complex. The absorbance of this complex at 593 nm shows the reducing power of samples.[14] The reduction of IC_{50} value after loading on column show reducing power of sample increased by using this type of column.

The results of ABTS assay have the same trend as DPPH and FRAP assays and antioxidant activity increased by loading on column (Table 1)

In nitric oxide radical scavenging assay, nitric oxide radicals ($NO^{\bullet}$) were produced by sodium nitroprusside, capable to interacts with oxygen and produce nitrite ions (NO^{-}).[16] The results of Nitric oxide scavenging ability of 200 µg/mL concentration of AFBI was 97.04 ± 0.69 after using amberlit column for anthocyanin purification, AFBI exhibited high nitric oxide scavenging ability, as reactive nitrogen species play critical role in cardiovascular disease, antioxidant be effective in prophylaxis of cardiovascular diseases.[20] According to Table.1, decreased IC_{50} of DPPH and FRAP assay and increased nitric oxide scavenging ability in loaded sample exhibit the efficiency of amberlite column to purify phenolic compound as mentioned in previous study.[21] Furthermore, strong antioxidant activity of this fraction due to present of anthocyanin.[22] Previous study revealed there is a linear relationship between the values of the antioxidant activity and the anthocyanin quantity in blackberries, red raspberries, black raspberries and strawberries.[23] Anthocyanins able to scavenge free radicals by donate of phenolic hydrogen atoms.[24] Besides hydrogen donation, other mechanisms engaged by anthocyanin are metal chelation, and protein binding.[25] Anthocyanins as powerful antioxidant prevent important biological macromolecule against oxidative stress. *In vivo* study confirm that dietary consumption of blueberry Black raspberry anthocyanin protect blood cells from free radicals and oxidative stress significantly.[26,27] So, it reveals the critical role of anthocyanin against reactive oxygen species cellular events such as cellular aging, mutagenesis, carcinogenesis, and coronary heart disease which may be mediated by destabilization of membranes, DNA damage and oxidation of low-density lipoprotein (LDL).[28]

The protective effect of AFBI on lipid peroxidation was determined by the ferric thiocyanate method (FTC). peroxides is a product of linoleic acid peroxidation, which able to oxidized Fe^{2+} to Fe^{3+}.The complex of Fe^{3+} ion with SCN had a maximum absorbance at 500 nm.[20]

As shown in figure1. The percentages of inhibition are about 39% in first day and 95% after 10 days. The inhibition percentage of lipid peroxidation was observed 66.863% for BHT and 64.06% for Vit C in the tenth day. According to these, it seems that AFBI is more effective than BHT and Vit C to protect lipid from peroxidation.

Lipid peroxidation assay exhibited the strong protective effect of AFBI on lipid peroxidation after ten days and there is significant difference with BHT and Vit C and AFBI.

Lipid peroxidation is results of free-radicals attack and oxidation of polyunsaturated fatty acids (PUFAs) which participate in membrane composition.[20-29] It is assumed that lipid oxidative damages related to change genes involved in developmental processes such as differentiation and aging and different disease.[30] So the ability of AFBI to prevent lipid peroxidation is promising to modify age related complications.

CONCLUSION

Our research shows the high antioxidant activity of *Berberis integerrima* Bunge fruits. The high level of anthocyanins content suggested that these fruits considered as appropriate source of functional antioxidant and improve human health. Further studies help to reveal more biological effects.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

ABBREVIATION USED

AFBI: Anthocyanin fraction of *Berberis integerrima* Bunge; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **ABTS:** 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); **FRAP:** Ferric Reducing Antioxidant Power; **NO:** Nitric Oxide; **TFA:** Trifluoroacetic acid; **MW:** Molecular Weight; **TPTZ:** 2,4,6-Tris(2-pyridyl)-s-triazine; **DNA:** Deoxyribonucleic Acid; **LDL:** Low-density lipoprotein; **$FeCl_3$:** Ferric chloride.

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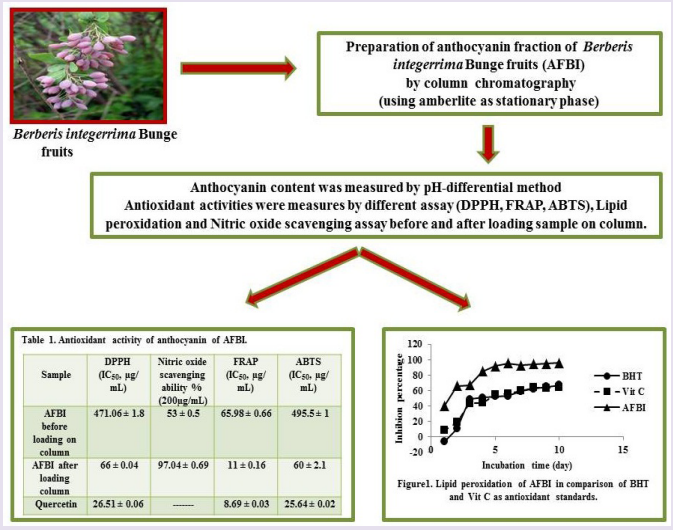
GRAPHICAL ABSTRACT



SUMMARY

- *Berberis integerrima* belongs to Berberidaceae comprise various secondary metabolites which candidate it as therapeutic herb in different disease. This study provides isolation of anthocyanin fraction of *Berberis integerrima* Bunge fruit by column chromatography. Anthocyanin content was measured by PH-differential method and antioxidant activities of this fraction quantified by various methods (DPPH, FRAP, ABTS) before and after loading on column as well as lipid peroxidation assay. Determination of antioxidant activities using different methods is worthwhile to realize the mechanism of antioxidant activities.

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