Original Article

# In Vitro Screening for Antioxidant Activity and Cancer Suppressive Effect of Blackberry (Morus Nigra)

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## Abstract

Anthocyanins are natural pigments widely spread in nature. Anthocyanin color molecules are a subclass of flavonoids. They are responsible for red, purple, and blue colors of many flowers, fruits and vegetables. Fruits and berries are sample sources of anthocyanins in nature. In many researches, the positive effects of fruits and berries intake on human health have been reported. Anthocyanins are considered to contribute to the healthiness of fruits and berries for their antioxidant, anti-carcinogenic, anti-inflammatory, and anti-angiogenic properties. The aim of the present study was to investigate the scavenging capacities towards super oxide anion radicals, and nitrite radicals and reducing power of anthocyanins extracted from Blackberry (Morus nigra) as a potential source of natural functional substances for use as dietary antioxidants. For superoxide anion radical assay, the superoxide anion radicals were generated by a pyrogallol auto oxidation system, Nitric oxide radical inhibition was done by using Griess Illosvoy reaction and its reducing power was determined according to the Oyaizu method. At least, all samples showed a potential antioxidant capacity that increased proportionate to the concentration of extracts. In this study, anthocyanin pigment was extracted from Blackberry by soaking and wetting in ethanol (۱٪acidified ).

**Keywords**:antioxidant ,c ancer (neoplasm), blackberry

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## Introduction

Epidemiological studies demonstrate that food can have beneficial effects on human health in addition to its nutritional value. In recent years, research in this area has focused on the detection of antioxidants in food, because there is evidence that they could play an important role in the prevention of several illnesses such as cancer and cardiovascular diseases as well as in the retardation of the aging process [۱]. Free radicals are unstable molecules that include a hydrogen atom, nitric oxide (NO] and molecular oxygen ($O_۲$). They are naturally produced in the body as a result of chemical reactions during normal cellular processes. Reactive oxygen species (ROS), sometimes called active oxygen species, are various forms of activated oxygen which include free radicals such as super oxide ions ($O^{۲-}$) and hydroxyl radicals (OH), as well as non free-radical species such as hydrogen peroxide ($H_۲O_۲$). In living organisms, various ROSs can be produced in different ways, including normal aerobic respiration, stimulated poly morphonuclear leukocytes and macrophages, and peroxisomes which appear to be the main endogenous sources of most of the oxidants produced by cells. Exogenous sources of free radicals include tobacco smoke, ionizing radiation, certain pollutants, organic solvents, and pesticides. In an attempt for free radicals to stabilize, they attack other molecules in the body potentially leading to cell damage and triggering the formation of another free radical resulting in a chain reaction. These reactive oxygen species have been implicated in certain chronic and ageing diseases, including cancer, malaria, rheumatoid arthritis, cataracts, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes and neurodegenerative diseases (Parkinson's and Alzheimer's diseases). Free radicals can also cause lipid peroxidation in foods, which leads to their deterioration. Oxidized polyunsaturated fatty acids may induce aging and carcinogenesis. When produced in excess, ROSs can cause tissue injury. However, tissue injury can itself cause ROS generation. Nevertheless, all aerobic organisms, including human beings, have defense mechanisms against antioxidants that protect them against oxidative damages and repair enzymes to remove or repair damaged molecules. However, this natural antioxidant mechanism can be inefficient, and

hence dietary intake of antioxidant compounds is important. Recent reports indicate that there is an inverse relationship between dietary intake of antioxidant rich foods and the incidence of human diseases [۲]. Increasing intake of dietary antioxidants may help to maintain an adequate antioxidant status and, therefore, the normal physiological function of a living system [۳]. Antioxidants are compounds that help to inhibit the many oxidation reactions caused by free radicals such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite thereby preventing or delaying damage to cells and tissues [٤]. Fresh fruits and vegetables, therefore not their industrial byproducts, are rich in antioxidants [٥] such as polyphenols, ascorbic acid, tocopherols, and carotenoids [٦-٩]. Polyphenols in fruits and vegetables include mainly flavonoids (flavonols, flavones, flavanones, isoflavones, flavanols, and anthocyanins), and phenolic acids (hydroxybenzoic and hydroxycinnamic acids) [٨]. Antioxidants are substances that are able to prevent or retard oxidation of lipids, proteins and DNA; and to protect the compounds or tissues from damage caused by oxygen or free radicals. Therefore, their health promoting effects reduce the risk of various diseases [٨].

Anthocyanins which are highly colored substances found in plants are possible for use in food, nutraceutical and pharmaceutical preparations for having most of the red, purple and blue colors and have a high potential as colorants because of their low toxicity [١٠]. Blackberries are a good source of anthocyanins in which the anthocyanin contents were reported to be ٦٧,٤–٢٣٠ mg/١٠٠ g fresh weight [١١]. The aim of the present study was to investigate the scavenging capacities towards superoxide anion radicals and nitrite radicals and the reducing power of the black berry juice as a potential source of natural functional substances for use as dietary antioxidants.

## Materials and Methods

### Sample preparation

Samples of *Berries* were obtained locally. They were washed with distilled water and kept frozen at–١٨ °C until use. The experiments were done in December ٢٠٠٦ to February ٢٠٠٧ at the biochemistry lab in Urmia University.

### Extraction of anthocyanins

Extraction was carried out by Chiriboga and Francis method [١٢]. Briefly, after taking the samples out of the freezer, they were left at room temperature for ٣٠ minutes to defrost. Then ١٠٠٠ grams from each sample was put into a mixer and was mixed for ١٠ minutes after adding ethanol solvent. Then, the products were filtered in Bochner funnel vacuum and Wattman filter (grade١), the remains of each mixture left on the filter paper was washed again with the above mentioned solvent and filtered again to get a Clear liquid. The filtered product then was placed in a balloon container within a vacuum evaporator at ٣٥ °C in order for the ethanol-acid solvent to separate. The balloon container was separated from the vacuum evaporator, and distilled water was added to dissolve the concentrated extract, which was formed at the bottom of the balloon container. The product was then transferred to a ١٠٠٠ ml container and after being expanded to ١٠٠٠ ml using distilled water, was centrifuged at ٨٠٠٠ rpm. Afterwards, the supernatant was separated and kept for further analysis.

### Determination of total anthocyanin content in Blackberry juice

The total content of monomeric anthocyanins in Blackberry juice was determined using the pH-differential method [١٣]. Absorbance was measured in a spectrophotometer at ٥١٠ and ٧٠٠ nm, in buffers at pH=١,٠ and ٤,٥, using A= {(A٥١٠–A٧٠٠) pH=١,٠ – (A٥١٠–A٧٠٠) pH=٤,٥} with the molar absorption coefficient of cyanidin-٣-glucoside of ٢٩ ٦٠٠ mol–١ L cm–١. The result was expressed as milligram of cyanidin-٣-glucoside equivalents (CGE) per gram of Blackberry juice.

### Scavenging capacity towards superoxide anion radicals

For one superoxide anion radical assay, the superoxide anion radicals were generated by a pyrogallol autoxidation system [١٤]. A volume of ٩ mL of Tris-HCl buffer solution (٥٠ mmol/L, pH=٨,٢) was added into a test tube, and the test tube was incubated in a water bath at ٢٥ °C for ٢٠ minutes. A volume of ٤٠μL of pyrogallol solution (٤٥ mmol/L of pyrogallol in ١٠ mmol/L of HCl), which was also pre-incubated at ٢٥ °C, was injected to the above test tube with a micro liter syringe and was mixed up. The mixture was incubated at ٢٥ °C for ٣ minutes and then a drop of ascorbic acid was dripped into the mixture promptly to terminate the reaction. The absorbance at ٤٢٠ nm marked as A٠ was measured ٥ minutes later (A٠ denotes the speed of pyrogallol autoxidation). The A١ autoxidation speed was obtained applying the aforementioned method and

along with adding a certain concentration of Blackberry's anthocyanin extract into the Tris-HCl buffer solution. Simultaneously, a blank control of reagent was obtained as A٢. The scavenging percentage was calculated according to the following formula:

Scavenging percentage= {A٠–(A١–A٢)} × ١٠٠/A٠.

**Nitric oxide radical inhibition assay**

Nitric oxide radical inhibition can be estimated by using Griess Illosvoy reaction [١٥]. In this investigation, Griess Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (٠,١٪ w/v) instead of ١-napthylamine (٥٪). The reaction mixture (٣ ml) containing sodium nitroprusside (١٠ mM, ٢ ml), phosphate buffer saline (٠,٥ ml) and Blackberry's anthocyanin extract (١٠ µg to ١٦٠ µg) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was mixed with ١ ml of sulfanilic acid reagent (٠,٣٣٪ in ٢٠٪ glacial acetic acid) and allowed to stand for ٥ min for completing diazotization. Then, ١ ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for ٣٠ min at ٢٥°C. A pink coloured chromophore is formed in diffused light. The absorbance of these solutions was measured at ٥٤٠ nm against the corresponding blank solutions.

**Reducing power**

The reducing power of Blackberry's anthocyanin extract was determined according to the Oyaizu method [١٦]. Different concentration of Blackberry's anthocyanin extract (١٠٠ µg – 1000 µg) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at ٣٠٠٠ rpm for ١٠ min. The upper layer of the solution (٢,٥ ml) was mixed with distilled water (٢,٥ ml)and FeCl ٣ (٠,٥ ml. ٠,١٪) and the absorbance was measured at ٧٠٠ nm. Increased absorbance of the reaction mixture indicated increased reducing power.

**Statistical analysis**

Statistical analysis of the data was performed by ANOVA using Microsoft SAS. All experiments were repeated three times.

## Results

**Scavenging capacity towards superoxide anion radicals**

As shown in Figure ١, the inhibition effect of anthocyanin pigments of blackberry extract on the autoxidation of pyrogallol was relatively feeble at lower concentrations, but it exhibited strong inhibition activities at higher concentrations. In the range of test concentrations from ٠,٠٥ to ٠,٧٥ mg/ml, the maximum inhibition percentage was ٥٧,١٤٪. This indicates that anthocyanin pigment of blackberry juice has a strong inhibitory effect on the autoxidation of pyrogallol. In other words, it can scavenge the superoxide anion radicals generated by the pyrogallol autoxidation system effectively.

**Nitric oxide radical inhibition assay**

The scavenging of nitric oxide by Blackberry's anthocyanin extract was increased in a dose-dependent manner as illustrated in figure ٢.

**Reducing power**

Figure ٣ shows the reductive capabilities of anthocyanin pigments in blackberry extract. The reducing power of anthocyanin pigments in blackberry extract was potent and the power of the extract increased proportionate to the quantity of the sample.

## Discussion

Free radicals were of significant interest among scientists in the past decade. Their broad range of effects in biological systems has drawn the attention of many experimental works. It has been proved that these mechanisms may be important in the pathogenesis of certain diseases and ageing. There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases [١٧]. Many synthetic antioxidant components have shown toxic and/or mutagenic effects, which have shifted the attention towards natural antioxidants. Numerous plant constituents have proven to show free radical scavenging or antioxidants activity [١٨]. Flavonoids and other phenolic compounds (hydroxyl cinnamic derivatives, catechines etc) of plant origins have been reported as scavengers and inhibitors of lipid peroxidation [١٩]. Superoxide anion is an initial free radical and plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems [٢٠]. It can also react with nitric oxide and form peroxynitrite, which can generate toxic

compounds such as hydroxyl radical and nitric dioxide [۲۱]. We evaluated the scavenging capacity of Blackberry's anthocyanin extract on superoxide anion radicals by using a pyrogallol autoxidation system. Pyrogallol can autoxidate fast in alkali conditions and release superoxide anions, and, in return, the superoxide anions can accelerate the autoxidation. However, the superoxide anions can be scavenged by adding some scavenger or antioxidant, the autoxidation would thus be depressed. As mentioned above, the superoxide anions were generated by the oxidation of pyrogallol and the scavenging effects were expressed as the inhibition of pyrogallol autoxidation, so any substance existing in the reaction system that might have effects on the oxidation of pyrogallol might affect the test results. Since the Blackberry's anthocyanin extract was a crude extract, there might have been some substances that could enhance the oxidation of pyrogallol and thereby offset some inhibition effects. Wang & Jiao (۲۰۰۰) considered scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. According to their results, among different cultivars, the highest antioxidant capacity against superoxide radicals, Hydrogen peroxide, Hydroxyl radical and singlet oxygen belonged to Blackberry. Afterwards, strawberry, cranberry, raspberry and blueberry had the greatest capacities, respectively [۲۲]. Also, Wanga & Ballington (۲۰۰۷) examined Free radical scavenging capacity and antioxidant enzyme activity in deer berry (Vaccinium stamineum L.) and described that its high antioxidant capacity was related to antioxidant enzymes activity [۲۳]. Duan X et al., (۲۰۰۷) extracted anthocyanins from litchi (Litchi chinenesis Sonn.) fruit pericarp tissues. Anthocyanins were extracted and purified from litchi fruit pericarp and their antioxidant properties were investigated. Anthocyanins from litchi fruit pericarp strongly inhibited superoxide anions and hydroxyl radical. It was therefore suggested that anthocyanins could be beneficial in scavenging free radicals [۲٤]. Ioannis G et al., (۲۰۰۵) examined the Scavenging Capacities of some types of wine and wine phenolic extracts. Results was indicated that all wine extracts exhibited scavenging capacity on hydroxyl radicals, superoxide radicals and singlet oxygen; Indicating that many wine phenolics may be active. However, they were different in their potency towards the three ROS tested. The most active extract towards superoxide radicals was rich in flavanols and anthocyanins. The characteristic phenolics of the most active wine extracts towards singlet oxygen were flavanols, flavonols and phenolic acids [۲۵]. Before them Saint-Cricq de Gaulejac et al (۱۹۹۹) had reported that wine fractions containing anthocyanins or oligomeric procyanidins were efficient scavengers of superoxide radicals [۲٦]. Nitric oxide radical inhibition study has proved that Blackberry's anthocyanin extract is a potent scavenger of nitric oxide. This nitric oxide generated from sodium nitro prusside reacts with oxygen to form nitrite. The extract inhibits nitrite formation by competing with oxygen to react with nitric oxide directly and also inhibits its synthesis. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide [۲۷]. Takanori Tsuda et al (۲۰۰۰) described the mechanism for the peroxynitrite scavenging activity by Anthocyanins. They showed that anthocyanins can function as a potent inhibitor of the formation of nitrated tyrosine in vitro, and clarified how Pelargonidin, which has one hydroxyl group on the B-ring, scavenges peroxynitrite by detection of the nitrated reaction products (٤-hydroxy-۳-nitrobenzoic acid) [۲۸]. They also demonstrated that cyaniding-۳-glucoside, which is one of the typical anthocyanins, has an antioxidant activity and protective effects against hepatic ischemia-reperfusion injury in vivo [۲۹]. Zhonggao Jiao et al (۲۰۰۵) studied antioxidant activities of total pigment extract from blackberries and stated that scavenging capacities of total pigment extract from blackberries towards nitrite were relatively feeble at lower concentrations, the scavenging activities became obviously stronger with an increasing concentration and showed a dose-dependent manner in the concentration range from ۰,۱ to ۱,۵ mg/ml. In the range of test concentration from ۰,۱ to ۲,۵ mg/ml, the maximum scavenging percentage was ۹۸,۹٤ %( ۳۰). Pergola Carlo et al (۲۰۰٦) showed nitric oxide biosynthesis inhibition by blackberry's anthocyanin extract. Their studies demonstrated that a part of anti inflammatory activity of blackberry extract was related to nitric oxide-produced inhibition by cyaniding-۳-glucoside that it is the major anthocyanin in blackberry extract. It seems that the mechanism of this inhibition is related to enzymes expression and activity [۳۱]. For the measurements of the reductive ability, we investigated the $Fe^{3+}$ to $Fe^{2+}$ transformation in Blackberry's anthocyanin extract using the method of Oyaizu et al [۱٦]. The reducing power increased with increasing the amount of extract. The reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity [۳۲]. The phenolic compounds may contribute directly to anti

oxidative action [۳۳]. Bae & Suh (۲۰۰۷) studied antioxidant activities of five different mulberry cultivars in Korea. The high value of reducing power indicates that some compounds in mulberry extract are electron donors which can react with free radicals to convert them into more stable products and to terminate radical chain reactions [۳٤]. Duan X et al., (۲۰۰۷) extracted anthocyanins from litchi (Litchi chinenesis Sonn.) fruit pericarp tissues. Anthocyanins were extracted and purified from litchi fruit pericarp and their antioxidant properties were investigated. Anthocyanins were found to have an excellent reducing power. It suggests that the anthocyanins have a strong electron-donating capacity. It is therefore suggested that anthocyanins could be beneficial in scavenging free radicals and reducing lipid peroxidation of litchi fruit pericarp [۲٤]. Yi Z et al (۲۰۰۷) examined In vitro antioxidant and antimicrobial activities of the extract of Pericarpium Citri Reticulatae of a new Citrus cultivar and its main flavonoids and reported that all samples showed some degrees of antioxidant activities in all the tested methods. Similarly, the reducing power of samples tested increased with increasing the amount [۳٥]. Tsai et al (۲۰۰٤) examined the reducing power of Anthocyanins in mulberry wine with FRAP method. They showed the antioxidant activity of Anthocyanins, too [۳٦]. This result indicates that anthocyanin pigments present in blackberry juice could be partly responsible for the beneficial effects. Compelling evidence indicates that increased consumption of dietary antioxidants or fruits and vegetables with antioxidant properties may contribute to the improvement in quality of life by delaying onset and reducing the risk of degenerative diseases associated with aging [۳۷،۳].

## Conclusion

Oxidative damage to cellular components such as lipids and cell membranes by free radicals and other reactive oxygen species is believed to be associated with the development of a range of degenerative diseases including heart diseases, cancer, inflammation, arthritis, immune system decline, brain dysfunction and cataracts [۲۱, ۳۹-٤۲]. This research has demonstrated that anthocyanin pigment in blackberry, which is commonly used as a natural food colorant, is also an excellent natural antioxidant and a free radical scavenger. Thus, it may play an important role in the prevention of human diseases related to oxidative damage. Furthermore, the anthocyanin pigment in blackberry has also exhibited a strong scavenging activity towards nitrite and thereby prevents the formation of nitrosamine and reduces the carcinogenesis induced by nitrosamines. Therefore, the anthocyanin pigment in blackberry is a natural, edible colorant with excellent antioxidant properties and health benefits and seems applicable in both healthy food and medicine.

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