Journal of Medicinal Plants Research Vol. 6(1), pp. 101-107, 9 January, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR11.1171
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

In-vitro free radical scavenging activities of anthocyanins from three berries

Haitian Zhao[1], Zhenyu Wang[1,2]*, Cuilin Cheng[1], Lei Yao[1,3], Lu Wang[1], Weihong Lu[1], Xin Yang[1] and Fengming Ma[1]

[1]School of Food Science and Engineering, Harbin Institute of Technology, 73 HuangHe Road, NanGang District, Harbin 150090, China.
[2]School of Forestry, Northeast Forestry University, 26 HeXing Road, DongLi District, Harbin 150040, China.
[3]National Research Center of Soybean Engineering and Technology, Northeast Agriculture University, 201 GongBin Road, XiangFang District, Harbin 150030, China.

Accepted 20 September, 2011

The purpose of this study was to evaluate the antioxidant and free radical scavenging properties of anthocyanin extracts from three berries (*Lonicera caerulea* var. edulis, *Vaccinium uliginosum* Linn. and *Rubus idaeus*). The anthocyanins extracted were found to show remarkable scavenging activity on 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical, hydroxyl radical (•OH) and superoxide anion radical ($\bullet O_2^-$). The effect of these three anthocyanins on their reducing power increases in a dose dependent manner. The results obtained in the present study indicate that the three anthocyanins can be a potential source for natural antioxidant activity.

Key words: Anthocyanin, berry, free radical, antioxidant activity.

INTRODUCTION

Free radicals and other oxygen-derived species are constantly generated *in vivo*, both by "accidents of chemistry" and for specific metabolic purposes (Halliwell, 1994). Free radicals are beneficial to the human body when present in correct amounts as they can safeguard the body from hazardous chemicals and other elements. However, excessive levels can lead to pathology as a result of severe oxidative damage to biological molecules, in particular, DNA, lipids and proteins (Gutteridge, 1994). Free radical-induced oxidative damage has been linked with a number of pathological events including coronary heart disease, cancer and aging (Halliwell, 1996).

Antioxidants have lots of important application because of their positive effects as health promoters for cardiovascular problems, atherosclerosis, treatment of cancers, and the ageing process (Packer et al., 1999). A number of synthetic antioxidants have been produced and added to food products. The advantage of these synthetic antioxidants is that they are very cheap and effective to produce. However, they remained questions of toxicity issues associated with their use (Valentão et al., 2002). Accordingly, much attention has been focused on the use of natural antioxidants. These natural antioxidants would be effective in protecting the human body from the oxidative damage by free radicals while avoiding the potential toxicity of synthetic antioxidants (Karimi et al., 2010).

Anthocyanins are secondary plant metabolites and are found in high concentrations in most berries. On the other hand, anthocyanins are members of the extensive class of phenolic compounds collectively named flavonoids. They are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts (Brouillard et al., 1982). The antioxidant properties of anthocyanins have been related in a number of reports in the literature (Radovanović et al., 2010; Tsuda et al.,

*Corresponding author. E-mail: wzy2190001@yahoo.cn. Tel: +86-451-86282909. Fax: +86-451-86282909.

1996; Einbond et al., 2004). Different compositions of anthocyanins are present in the extracts of various berries. Moreover, the altitude and conditions of cultivation are important factors with regard to the anthocyanin content of berries (Viskelis et al., 2009).

In our previous work, we found that three berries, *Lonicera caerulea* var. edulis (blue honeysuckle), *Rubus idaeus* (red raspberry) and *Vaccinium uliginosum* Linn. (blueberry) indigenous to the Greater Higgnan Mountains in northeast China, were rich in anthocyanins. Anthocyanins content of *L. caerulea* var. edulis was the highest (368 ± 8.7 mg/100 g fresh weight) (Fan et al., 2011). However, there is little information concerning on the antioxidant and free radicals scavenging properties of anthocyanin from the berries – *L. caerulea* var. edulis and *R. idaeus.* The purpose of the present study is to evaluate the different anthocyanins from these two berries along with that of *V. uliginosum* Linn. These berries were evaluated for their antioxidant activities against radicals of 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), hydroxyl radicals (•OH) and superoxide radicals ($•O_2^-$), and to assess whether these species could be sources of natural antioxidants for pharmaceutical and food applications.

MATERIALS AND METHODS

Chemicals and instruments

Tris (hydroxymethyl) aminomethane (Tris), 2,2-Diphenyl-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) were purchased from Sigma Chemical Co., USA. The 30% hydrogen peroxide, sodium salicylate, pyrogallol, and other chemicals in the studies were of analytical grade purchased from local suppliers (Sinopharm Chemical Reagent Beijing Co., Ltd, China). All absorbance were measured by a UV–Vis spectrophotometer (Shimadzu UV-2000, Japan).

Plant material

L. caerulea var. edulis, *V. uliginosum* Linn. and *R. idaeus* were harvested from the Greater Higgnan Mountains. Voucher of specimens were performed by Prof. Shaoquan Nie, School of Forestry, Northeast Forestry University, China.

Preparation of the anthocyanin

The berry fruits were extracted twice with an ultrasonic apparatus (KQ-500DB, Kunshan Ultrasonic Instruments Co., Ltd., Kunshan, China). The ultrasonic extraction was performed with ethanol/water/hydrochloric acid (60:40:0.1, v/v/v) of solid-liquid ratio 1:5 (g:ml), under 400 W extracting power at room temperature for 1 h.

The filtrates were combined and concentration by vacuum evaporation. The concentrated extracts were loaded onto an X-5 macroporous resin. The X-5 resin was washed with distilled water, and subsequently the absorbed anthocyanins were eluted with 60% ethanol. The filtrate of the extract was concentrated and the extract powder was obtained by freeze drying. The anthocyanin extract powders were stored at 4°C until required. Prior to assay, extracts were dissolved to generate the required concentrations.

Determination of total anthocyanin content

The total anthocyanin content was measured using a modified pH differential method (Lee et al., 2005). Absorbance of anthocyanin at 510 nm and 700 nm in different pH buffers (pH 1.0 and 4.5) were measured respectively. Absorbance readings were converted to total mg of cyanidin 3-glucoside per 100 g fresh weight of strawberry using the molar extinction coefficient of 26,900 and absorbance of A. Calculate anthocyanin pigment concentration, expressed as cyanidin-3-glucoside equivalents, as follows:

$$\text{Anthocyanin pigment (cyd-3-glu, mg/ml)} = \frac{A \bullet Mw \bullet DF}{\varepsilon \times 1}$$

A = [(A_{510} - A_{700}) pH 1.0 - (A_{510}- A_{700}) pH 4.5]; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor; l = path length in cm.

DPPH radical scavenging activity

DPPH radical scavenging activity was measured according to the method of Blois et al. (1958) with slight modifications. Briefly, anthocyanins at various concentrations (0.1 ml) were mixed with 1.4 ml of a DPPH-methanol solution (0.1 mM). After the solution was incubated in the dark for 30 min at room temperature, the absorbance was measured at 517 nm. Ascorbic acid was used as the positive control. Scavenging activity of the DPPH free radical was measured as a decrease in the absorbance of DPPH. The inhibition ratio was calculated using the following equation:

$$\text{Percentage scavenging of DPPH} = [1-(A_1- A_2)/A_0] \times 100$$

Where A_0 is the absorbance of DPPH alone, A_1 is the absorbance of DPPH + anthocyanin and A_2 is the absorbance of the anthocyanin only. All samples were done in triplicate and the data are presented as mean ± SD.

ABTS radical scavenging activity

ABTS radical cation scavenging activity was determined according to the method of Bao et al. (2005) with slight modifications. The ABTS solution was diluted with ethanol to a final absorbance of the control of 0.70±0.01 at 734 nm before use. The anthocyanin solution (0.1 ml) was mixed with 1.9 ml of the ABTS radical solution. The control solution was also prepared by replacing 0.1 ml deionized water with the anthocyanin solution. The mixture was vortexed for 10 s and then incubated in the dark at room temperature for 5 min. The absorbance at 734 nm was immediately recorded.

The percentage of ABTS radical-scavenging activity of anthocyanin was calculated using the following equation:

$$\text{Percentage scavenging of ABTS} = [A_0 - (A_1 - A_2)]/A_0 \times 100$$

A_0 is the absorbance of the control solution (containing only ABTS); A_1 is the absorbance of the ABTS solution containing anthocyanin; and A_2 is the absorbance of the sample anthocyanin solution without ABTS. Ascorbic acid was used as the positive

control. All assays were performed in triplicate.

Hydroxyl radical (·OH) assay

Assay for the hydroxyl radical (•OH) was performed using the methods of Su et al. (2009) with slight modifications. Hydroxyl radicals were generated by the Fenton reaction. The reaction system contained 0.5 ml $FeSO_4$ (9 mM), 1.0 ml H_2O_2 (8.8 mM), 1.0 ml of various concentrations of the anthocyanin solution and 0.2 ml salicylic acid (9 mM). The total mixture was incubated at 37℃ for 1 h and the absorbance of the mixture was then recorded at 510 nm. Ascorbic acid was used as the positive control. The scavenging activity was calculated using the following equation:

Scavenging rate (%) = $[1-(A_1-A_2)/A_0] \times 100$

Where A_0 is the absorbance of the control (without anthocyanin), A_1 is the absorbance of the polysaccharide addition and A_2 is the absorbance without salicylic acid.

Superoxide radical (·O_2^-) assay

Assay for the superoxide anion radical (•O_2^-) was performed using the methods of Marklund et al. (1974) with slight modifications. In brief, 0.2 ml various concentrations of anthocyanins were added to 5.7 ml of 50 mM Tris-HCl buffer (pH 8.2). The mixture was incubated at 25℃ for 10 min and then 0.1 ml of 6 mM pyrogallol (dissolved in 10 mmol/L HCl) was added. The absorbance of the reaction mixture at 320 nm was measured at 5 min. For comparison, the •O_2^- scavenging activity of ascorbic acid was also tested. Scavenging activity was calculated as follows:

Scavenging rate (%) = $[1-(A_1-A_2)/A_0] \times 100$

Where A_0 is the autoxidation rate of pyrogallol for control (the change of the absorbance), A_1 is the oxidation rate of pyrogallol for samples, and A_2 is the absorbance of the sample blank.

Statistical analysis

All experiments were performed in triplicate, and results are expressed as mean ± standard deviation and analyzed by SPSS 17.0 for Windows software package.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

DPPH scavenging activity has been used by various researchers as a quick and reliable assay to assess the *in vitro* antioxidant activity of plant extracts (Choi et al., 2002). The scavenging activities of DPPH exerted by anthocyanins as well as ascorbic acid are presented in Figure 1. All three anthocyanins demonstrated strong DPPH radical scavenging activities. Ascorbic acid, included as a positive control, showed the lowest median effective concentration (EC_{50}) 6±0.4 µg/ml. Rankings for the scavenging activity of the three berries were, *L. caerulea* var. edulis (EC_{50} = 30±2 µg/ml) > *V. uliginosum* Linn. (35±2 µg/ml) > *R. idaeus* (EC_{50}= 50±3 µg/ml). At a concentration greater than 200 µg/ml, anthocyanins showed higher scavenging effects than ascorbic acid. The robust scavenging capacity of anthocyanin on DPPH was possibly due to the presence of phenolic compounds which could act as a hydrogen donor antioxidant.

ABTS radical scavenging activity

ABTS is a well-known nitrogen-centred synthetic radical and is widely used to determine antioxidant activity. The ABTS radical is generated by oxidation of ABTS with potassium persulphate. When antioxidants are added to the ABTS radical, it is converted to a non-radical form (Debnatha et al., 2011). Dose-response relationships for the three antocyanins and ascorbic acid on scavenging of the ABTS radical are shown in Figure 2. Our results reveal that all three antocyanins are capable of demonstrating strong scavenging activities on the ABTS radical, being only slightly lower than that observed for ascorbic acid (EC_{50} = 42±4 µg/ml). Among the three anthocynins, *L. caerulea* var. edulis showed the highest scavenging activity (EC_{50} = 80±3 µg/ml) followed by *V. uliginosum* Linn. (EC_{50} = 100±5 µg/ml) and *R. idaeus* (EC_{50} = 104±4 µg/ml). At a concentration above 400 µg/ml, the scavenging rate of anthocyanins can reach 90%.

Hydroxyl radical (·OH) inhibition activity

The hydroxyl radical is the most reactive of the free radicals and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal–ions, such as copper or iron. Hydroxyl radicals can damage adjacent biomolecules such as all proteins, DNA, polyunsaturated fatty acid (PUFA), nucleic acids, and almost any biological molecule it contacts (Aruoma, 1998). The resultant damage is believed to contribute to the aging process, cancer and several other diseases (Zhu et al., 2006). Therefore, removal of the hydroxyl radical represents one of the most effective defenses marshaled by the body.

Figure 3 shows the hydroxyl radical-scavenging effects of anthocyanins and ascorbic acid. The scavenging effect of anthocyanin on hydroxyl radicals was concentration-dependent. Above a concentration of 0.4 mg/ml, the scavenging rates of three anthocyanins achieve 70%. Based on the EC_{50} of the assay, the ranking of •OH scavenging activity of anthocyanins from the three berries were *V. uliginosum* Linn (0.017±0.002). > *L. caerulea* var. edulis (0.028±0.002) > *R.* idaeus (0.048±0.02) > ascorbic

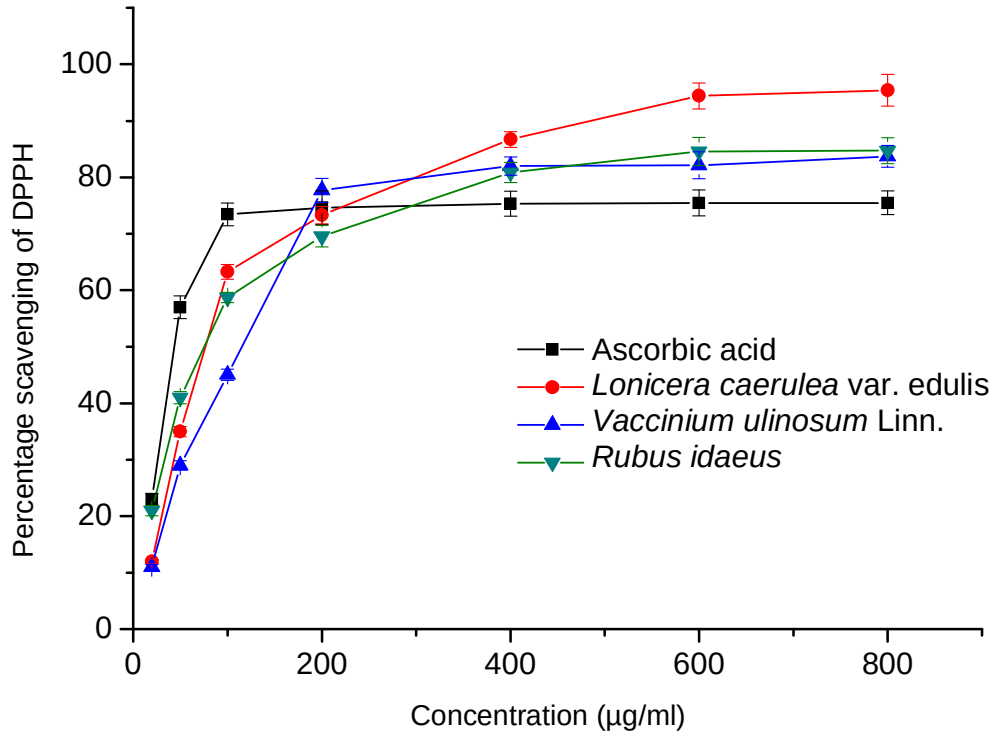

Figure 1. DPPH radical scavenging activity of anthocyanins from three berries and ascorbic acid.

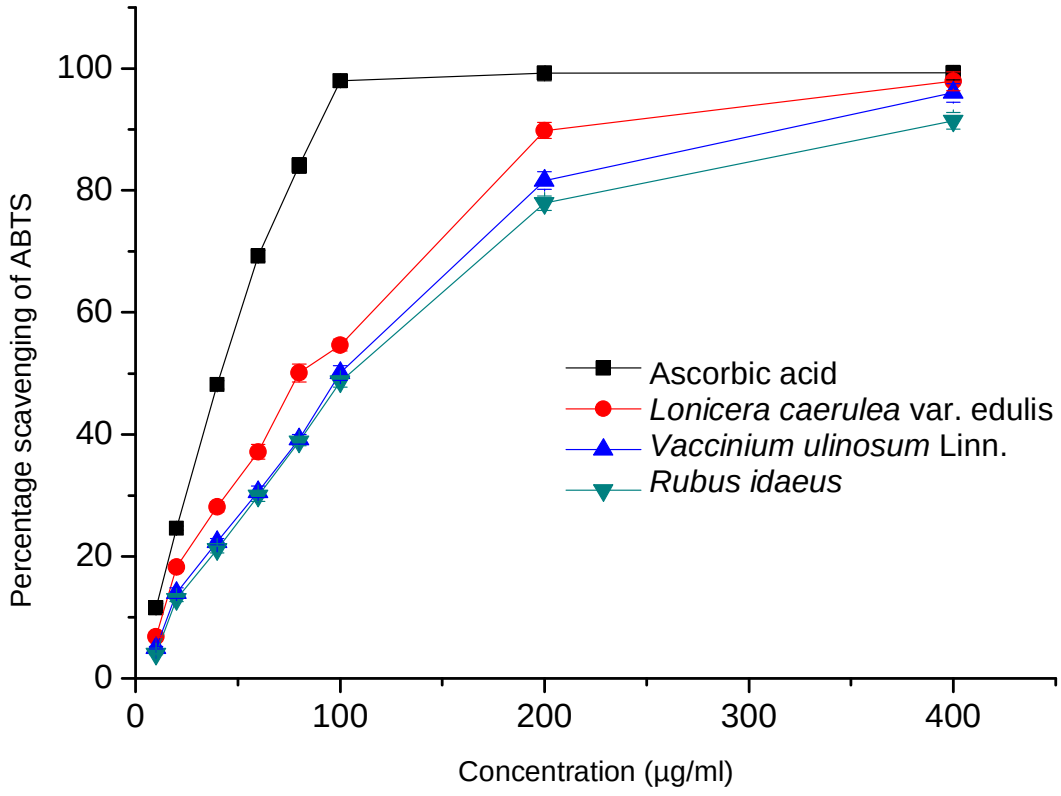

Figure 2. ABTS radical scavenging activity of ascorbic acid and anthocyanin of three berries.

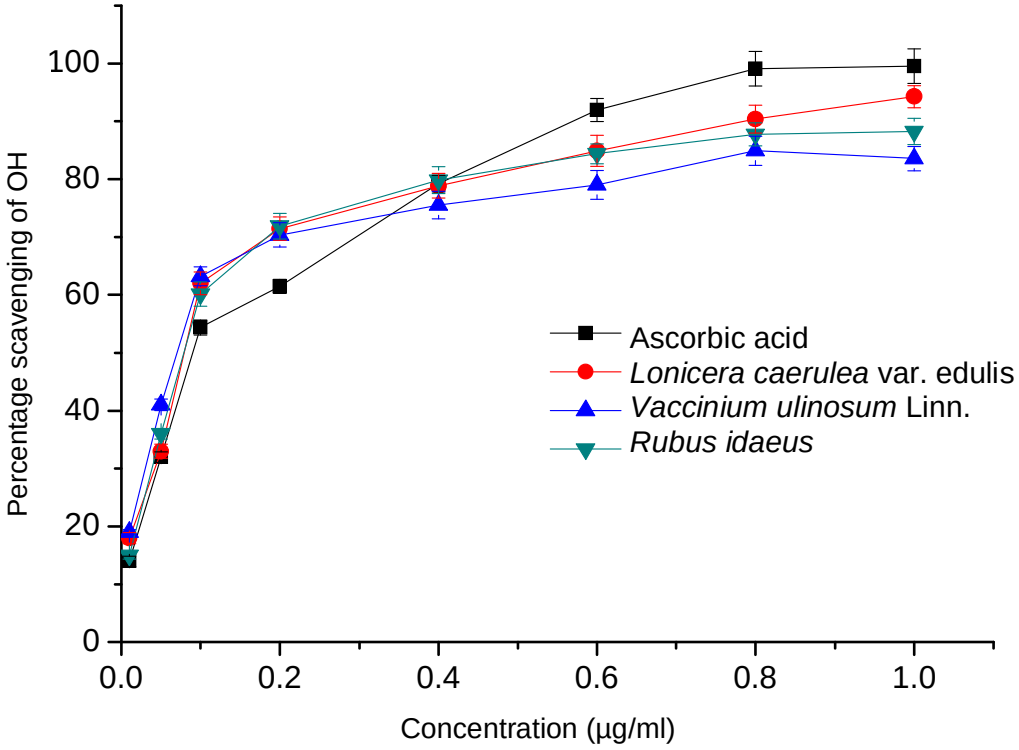

Figure 3. Hydroxyl radical scavenging activity of ascorbic acid and anthocyanin of three berries.

acid (0.068±0.04).

Superoxide anion radical (•O_2^-) inhibition activity

Superoxide anion plays an important role in the formation of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which can induce oxidative damage in lipids, proteins and DNA (Pietta, 2000). Superoxide anion radicals are produced from molecular oxygen due to oxidative enzymes of body as well as via non enzymatic reactions such as autoxidation by pyrogallol, sulphite, adrenalin, or 6-hydroxy-dopamine (Misra et al., 1972).

In this study, the pyrogallol autoxidation assay was used to evaluate superoxide anion radical scavenging effects of anthocyanins with ascorbic acid used as a positive control. Scavenging abilities of the three anthocyanins on superoxide anion radicals are shown in Figure 4. Ascorbic acid showed an excellent degree of scavenging activity on the superoxide anion radical (EC_{50} = 0.23±0.22 mg/ml). The three anthocyanins were also effective scavengers of the superoxide anion radical, although they showed a relatively low inhibitory effect. The inhibitory effect of *L. caerulea* var. edulis (EC_{50} = 1.43±0.12 mg/ml) was slightly better than *V. uliginosum* Linn. (EC_{50} = 1.56±0.17 mg/ml) and substantially better than *R. idaeus* (EC_{50} = 2.12±0.11 mg/ml).

Conclusions

The free radical scavenging activities of the anthocyanins derived from *L. caerulea* var. edulis, *V. uliginosum* Linn and *R. idaeus* were investigated *in vitro*. Assessment methods included scavenging effects on the DPPH, ABTS, hydroxyl radicals (•OH) and super oxidant anion (•O_2^-). The results from these assays indicated that all the three anthocyanins are promising natural products that can function as free radical scavengers. Of the three berries tested, anthocyanins of *L. caerulea* var. edulis showed the best scavenging activity on DPPH, ABTS, and hydroxyl radicals, while its scavenging effect on super oxidant anion was slightly lower than *V. uliginosum* Linn. Different sources of anthocyanins possess differing degrees of free radical scavenging effects. This may be attributed to differences in their specific anthocyanin monomer composition.

According to the present findings, these anthocyanins might be used to provide a natural and effective free

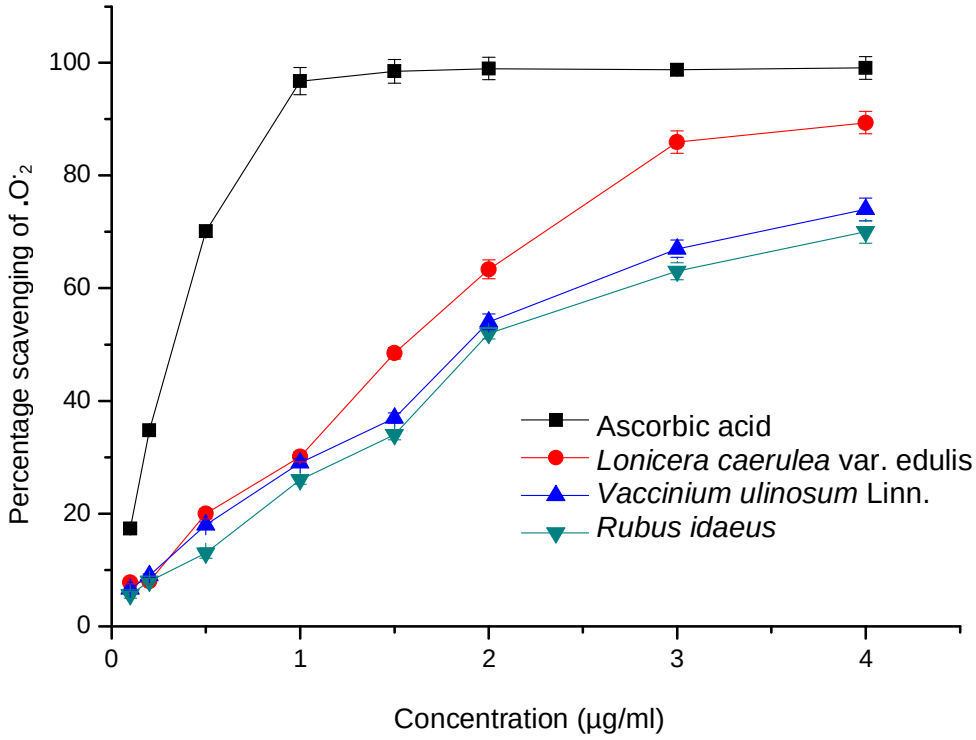

Figure 4. Superoxide anion radical ($\bullet O_2^-$) scavenging activity of ascorbic acid and anthocyanin of three berries.

radical scavenger food additive for use in humans. Further work on the composition and antioxidant activities of these anthocyanins as assessed *in vivo* is in progress.

ACKNOWLEDGEMENTS

We gratefully acknowledge the financial support from the National Natural Science Foundation of China (No.31170510 and 31000831), National Science Foundation for Post-doctoral Scientists of China (20090450478) and Natural Science Foundation of Heilongjiang Province (QC08C01).

REFERENCES

Aruoma OI (1998). Free radicals, oxidative stress, and antioxidants in human health and disease. J. Am. Oil Chem., 75: 199-212.

Bao J, Cai Y, Sun M, Wang GY, Corke H (2005). Anthocyanins, flavonols, and free radical scavenging activity of Chinese bayberry (*Myrica rubra*) extracts and their color properties and stability. J. Agr. Food Chem., 53: 2327-2332.

Blois MS (1958). Antioxidant determinations by the use of a stable free radical. Nature. 26: 1199-1200.

Brouillard R, Iacobucci G, Sweeny JG (1982). Chemistry of anthocyanin pigments. 9. UV-visible spectrophotometric determination of the acidity constants of apigeninidin and three related 3-deoxyflavylium salts. J. Am. Chem., 104: 7585-7590.

Choi CW, Kim SC, Hwang SS, Choi BK, Ahn HJ, Lee MY, Park SH, Kim SK (2002). Antioxidant activity and free radical scavenging capacity between Korean medicinal plants and flavonoids by assay-guided comparison. Plant Sci., 163(6): 1161-1168

Debnatha T, Park PJ, Nath NCD, Samad NB, Park HW, Lim BO (2011). Antioxidant activity of *Gardenia jasminoides* Ellis fruit extracts. Food Chem., 128: 697-703.

Einbond LS, Reynertson KA, Luo XD, Basile MJ, Kennelly EJ (2004). Anthocyanin antioxidants from edible fruits. Food Chem., 84: 23-28.

Fan ZL, Wang ZY, Liu JR (2011). Cold-field fruit extracts exert different antioxidant and antiproliferative activities in vitro, Food Chem., 129(2): 402-407

Gutteridge JM (1994). Biological origin of free radicals, and mechanisms of antioxidant protection. Chem-Biol. Interact., 91: 133-140.

Halliwell B (1994). Free radicals and antioxidants: a personal view. Nutr. Rev., 52: 253-265.

Halliwell B (1996). Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans, Free Rad. Res., 25: 57-74.

Karimi E, Oskoueian E, Hendra R, Jaafar HZE (2010). Evaluation of *Crocus sativus* L. Stigma phenolic and flavonoid compounds and its antioxidant activity. Molecules, 15: 6244-6256.

Lee JM, Durst RW, Wrolstad RE (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. J. AOAC Int., 88: 1269-1278.

Marklund S, Marklund G (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem., 47: 469-474.

Misra HP, Fridovich I (1972). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem., 247: 3170-3175.

Packer L, Rimbacha G, Virgilia F (1999). Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (*Pinus maritima*) bark, pycnogenol. Free Rad. B., 27: 704-724.

Pietta PG (2000). Flavonoids as antioxidants. J. Nat. Prod., 63: 1035-1042.
Radovanović B, Radovanović A (2010). Free radical scavenging activity and anthocyanin profile of Cabernet Sauvignon wines from the Balkan region. Molecules. 15: 4213-4226.
Su XY, Wang ZY, Liu JR (2009). *In vitro* and *in vivo* antioxidant activity of *Pinus koraiensis* seed extract containing phenolic compounds. Food Chem., 117: 681-686.
Tsuda T, Shigu K, Ohhim K, Kuwakishi S, Osuwu T (1996). Inhibition of Lipid Peroxidation and the Active Oxygen Radical Scavenging Effect of Anthocyanin Pigments Isolated from *Phaseoks vulgaris* L.. Biochem. Pharm., 52: 1033-1039.
Valentão P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML (2002). Antioxidative properties of cardoon (*Cynara cardunculus* L.) infusion against superoxide radical, hydroxyl radical, and hypochlorous acid. J. Agr. Food Chem., 50: 4989-4993.
Viskelis P, Rubinskiene M, Jasutiene I, Sarkinas A, Daubaras R, Cesoniene L (2009). Anthocyanins, antioxidative, and antimicrobial properties of American cranberry (*Vaccinium macrocarpon* Ait.) and their press cakes. J. Food Sci., 74: C157-161.
Zhu K, Zhou H, Qian H (2006). Antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with alcalase. Process Biol., 41: 1296-1302.