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The Stability and Antioxidant Activity of Anthocyanins from Blueberry

Bo Chu Wang, Rui He and Zhi Min Li*

Key Laboratory of Biorheological Science and Technology, Bioengineering College, Chongqing University, Chongqing 400044, PR China

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Summary

Anthocyanins from highbush blueberry (*Vaccinium corymbosum* L.) have tremendous potential as natural colorants and functional food with pharmaceutical purposes in food applications. To exploit the potential for food applications, the stability and antioxidant activity of anthocyanins present in blueberries have been studied. The results indicate that anthocyanins from blueberry were stable against the low pH (≤5.0), NaCl (0.125–0.500 mol/L), sucrose (0.584–2.336 mol/L) and preservative (sodium benzoate, 0.035–0.140 mol/L), but were sensitive to alkaline conditions (≥7.0), high temperature (≥80 °C), light (natural light), oxidizing agent (H_2O_2, 0.5–2.0 %) and reducing agent (Na_2SO_3, 0.005–0.040 mol/L). At concentrations of 25 and 50 μg/mL, anthocyanins from blueberry could protect ECV-304 cells against oxidative damage induced by H_2O_2. These results suggest that anthocyanins from blueberry can be regarded as a potential colorant for some acidic (pH≤5.0) food products and could be used as health food to prevent diseases arising from oxidative processes.

Key words: highbush blueberry, anthocyanins, stability, antioxidant activity

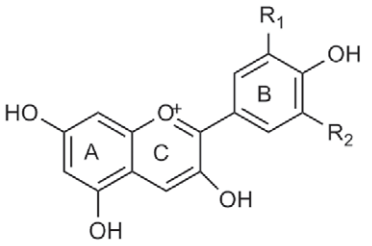
Introduction

Highbush blueberry (*Vaccinium corymbosum* L.) is a rich source of antioxidants among all fresh fruits and vegetables (*1*). It grows abundantly in the USA, Canada (*2*), and also in China. Blueberry is rich in anthocyanins (25–495 mg per 100 g) (*3*), which provide red, blue, yellow, purple and other colours to vegetables and fruits that contain them (*4*). Anthocyanins are glycosides and acylglycosides of anthocyanidins. There are six anthocyanidins commonly found in the nature (Fig. 1) (*3,5*).

Anthocyanins from blueberry have drawn interest for their potential as natural food colorants and as functional food with pharmaceutical purposes. Many studies have shown that anthocyanins are not only nontoxic and nonmutagenic, but have positive therapeutic properties (*6–10*). A number of their activities have been extensively reviewed (*3*), such as antioxidant (*11*), antiulcer (*12*), prevention of cardiovascular diseases (*13*), anticancer, antitumor and antimutagenic (*14*), antidiabetes (*15,16*), ocular (*17*), treatments for age-related diseases (*18*), and antibacterial activity (*19*).

Due to their attractive, bright colours, water solubility, along with their positive therapeutic effects, blueberry anthocyanins are considered as potential substitutes for synthetic colorant and usage in dietary supplements (*20*). However, several factors limit their possibilities as commercial colorant and diet therapy since anthocyanins have stability problems (*21*). Their stability has not been sufficiently studied yet. To provide some reliable data for exploitation of the resources in food applications, the effect of pH, temperature, light, oxidizing agent, reducing agent and common food additives (NaCl, sucrose and sodium benzoate) on the stability of anthocyanins, as well as their antioxidant activity were studied.

*Corresponding author; Phone: ++86 13 50 839 6242; Fax: ++86 23 6510 2507; E-mail: wangbc2000@126.com



Name	R_1	R_2
Pelargonidin	H	H
Cyanidin	H	OH
Delphinidin	OH	OH
Peonidin	H	OCH_3
Petunidin	OH	OCH_3
Malvidin	OCH_3	OCH_3

Fig. 1. Chemical structures of the most common anthocyanidins in the nature

Materials and Methods

Materials

Fresh highbush blueberries (*Vaccinium corymbosum* L.) were purchased from a local supermarket and identified at the College of Bioengineering, Chongqing University, Chongqing, PR China. Human umbilical vein endothelial cell, line ECV304, was obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, PR China. All chemicals used in the study were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade.

Extraction of anthocyanins

The extraction of anthocyanins from blueberry was adapted from the work done by Lohachoompol *et al.* (22) and Garcia-Viguera *et al.* (23). Briefly, blueberries were ground into puree at 5 °C. A mass of 100 g of the well blended puree was extracted using 750 mL of MeOH/water/acetic acid (25:24:1). The obtained mixture was filtered, and the residue was extracted twice more with the additional MeOH/water/acetic acid. The extract was combined and concentrated under vacuum at 35 °C. The crude extract was redissolved in 10 mL of 3 % (by mass per volume) formic acid in distilled water and purified using a C18 Sep-Pak cartridge. The anthocyanins eluted from the cartridge were concentrated under vacuum at 35 °C, lyophilized, preserved at –20 °C and used as required. The content of anthocyanins was measured by the pH-differential method described earlier (22).

Degradation of anthocyanins

The influence of key factors, including pH, temperature, light, oxidizing agent, reducing agent, NaCl, sucrose and preservative (sodium benzoate), on the stability of anthocyanins was studied.

The amount of the remaining anthocyanins was calculated using the following formula:

$$\text{Remaining rate of anthocyanins} = \left(\frac{\text{anthocyanin content before treatment}}{\text{anthocyanin content after treatment}}\right)\times 100 \quad /1/$$

In the pH study, buffers at five pH values (1.0, 3.0, 5.0, 7.0 and 9.0) were measured by a pHS-25 meter (Shanghai Precision Scientific Instruments Corporation, PR China) and prepared as follows: pH=1.0 (67.0 mL of 0.2 mol/L of hydrochloric acid and 25.0 mL of 0.2 mol/L of KCl), pH=3.0 (20.55 mL of 0.2 mol/L of Na_2HPO_3 and 79.45 mL of 0.1 mol/L of citric acid), pH=5.0 (51.50 mL of 0.2 mol/L of Na_2HPO_3 and 48.50 mL of 0.1 mol/L of citric acid), pH=7.0 (82.35 mL of 0.2 mol/L of Na_2HPO_3 and 17.65 mL of 0.1 mol/L of citric acid), and pH=9.0 (100 mL of 0.04 mol/L of barbituric acid and 1.65 mL of 0.2 mol/L of hydrochloric acid). The extract of anthocyanins was dissolved in different pH buffers (5 mg per 5 mL) in separate volumetric flasks and placed for 10 days at room temperature under the same conditions. The absorbance of each extract was detected every day at the same time using a 756 UV/VIS spectrophotometer (Shanghai Precision Scientific Instruments Corporation, PR China).

In the studies of temperature, light, oxidants, reductants, NaCl, sucrose, and degradation of preservatives, water solutions of anthocyanins were prepared as follows: a mass of 20 mg of anthocyanins was dissolved in 10 mL of citric acid/Na_2HPO_3 buffer (1 %, pH=3.0). The samples were diluted to appropriate concentrations, the initial absorbance of which was between 0.45 and 0.80.

In order to study the effect of temperature on the stability of anthocyanins, diluted samples were placed at room temperature, and at 40, 60, 80 and 100 °C for 2 h. Then the absorbance was determined at 30-minute intervals at 535 nm.

To study the degradation of anthocyanins under the influence of light, diluted samples were exposed to respectively natural light outdoors, natural light indoors and dark room. The absorbance of each extract was determined on the 2nd, 4th, 6th and 8th day.

To investigate whether oxidizing or reducing agents can affect the stability of anthocyanins, samples were exposed to different concentrations of H_2O_2 and Na_2SO_3. Diluted samples of anthocyanins were prepared with 0, 0.5, 1.0, 1.5 and 2.0 % of H_2O_2 and with 0, 0.005, 0.010, 0.020 and 0.040 mol/L of Na_2SO_3. The absorbance of each sample was determined after 1-hour exposure to an oxidizing or reducing agent at room temperature.

In order to study the influence of common food additives on the stability of anthocyanins, experiments were designed in the presence of NaCl, sucrose and sodium benzoate. Solutions of anthocyanins were prepared with 0, 0.125, 0.250, 0.375 and 0.500 mol/L of NaCl, with 0, 0.584, 1.168, 1.752 and 2.336 mol/L of sucrose, and with 0, 0.035, 0.070, 0.105 and 0.140 mol/L of sodium benzoate. The absorbance of each sample was determined after one-day exposure at room temperature.

Antioxidant activity of anthocyanins

ECV-304 cells were cultured in the RPMI-1640 medium supplemented with 10 % fetal bovine serum in cell culture incubator (5 % CO_2) at 37 °C. When monolayer cells were 80–90 % confluent, they were split into new flasks using 0.25 % trypsin and 0.02 % EDTA.

The control cells were cultured in the RPMI-1640 medium supplemented with 10 % fetal bovine serum for 25 h. To check whether anthocyanins could provide protection against oxidation damage generated with H_2O_2, the cells were cultured in the RPMI-1640 medium supplemented with 10 % fetal bovine serum or different concentrations (15, 25, 50 and 100 μg/mL) of anthocyanins for 24 h before exposure to 1 mmol/L of H_2O_2 for 1 h. The absorbance was determined at 570 nm using 3--(4,5 dimethylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide (MTT) method (*24*). The absorbance reflects the quantity of the living cells and determines the antioxidant activity of protecting ECV-304 cells against H_2O_2 damage. Morphological changes of ECV-304 cells were observed by optical microscope.

Statistical analysis

All results were expressed as mean value±standard deviation (SD) (*N*=3). The data analysis was carried out using SPSS 13.0 software. One way analysis of variance (ANOVA) was used to test the significance of differences between treatments. The results with $p<0.05$ were considered to be statistically significant.

Results and Discussion

With increased public concern about the security of synthetic colorants, the interest in natural pigment extracts is growing (*6*). The use of anthocyanins from blueberry as part of the human diet and as a health food has a long history (*2,3*). However, their usage as potential natural colorants and nutraceuticals is attracting attention. Factors affecting their applications in terms of stability and antioxidant activity are discussed in this study.

Total content of anthocyanins

The total anthocyanin content of the tested blueberries was (7.9±0.9) g of cyanidin 3-glucoside equivalent per kg of mass, the same as reported earlier (*22*).

Degradation studies

The effect of pH on the stability of anthocyanins

The absorbance of each buffer solution of anthocyanins determined every day is presented in Fig. 2. The results show that absorbance decrease was not significant when pH≤5.0, but anthocyanins degraded significantly when pH≥7.0. The percentage of the remaining anthocyanins was only 73.5 and 67.9 % at pH=7.0 and 9.0, respectively, after 10 days of exposure. This demonstrated that the anthocyanins were stable under low pH values (1.0, 3.0, 5.0) and unstable under alkaline conditions. The stability of anthocyanins strongly depends on their structure. At very low pH values (<3.0), anthocyanins in aqueous solution exist mostly in the red flavylium cation (AH^+) form. In detail, in the aqueous solution an equilibrium of four major anthocyanins: blue quinoidal base (A), AH^+, colourless carbinel (B) and colourless chalcone (C) exists as $A \leftrightarrow AH^+ \leftrightarrow B \leftrightarrow C$, which is controlled by the pH. At very low pH values, AH^+ is dominant. At higher pH values, AH^+ is transformed to B, further to C, and also to A with increasing pH values. The susceptibility

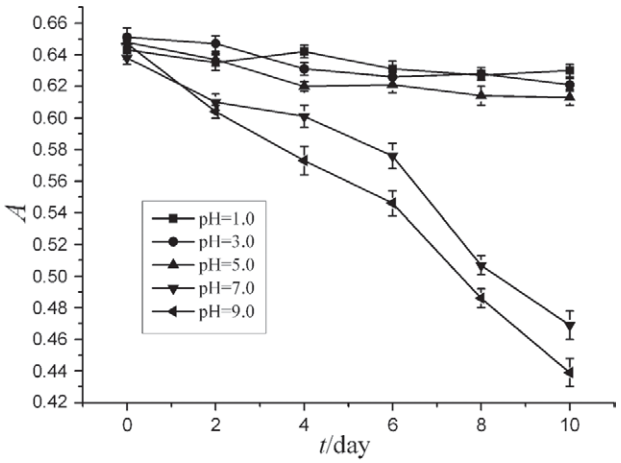


Fig. 2. Effect of the pH (1.0, 3.0, 5.0, 7.0, and 9.0) on the stability of anthocyanins. The absorbance of each extract was determined at 535 nm every day at the same time for 10 days. Each value is expressed as mean±S.D. (*N*=3)

of AH^+ towards degradation is extremely low (*25*). Hence, anthocyanins are most stable at low pH values.

The study of the effect of pH on the stability of anthocyanins indicated that they were susceptible to pH changes. These results indicate clearly that low pH values are needed to study the degradation of anthocyanins. This is consistent with the results of most published studies on the stability of anthocyanins. For instance, Kırca *et al*. (*26*) found that the stability of black carrot anthocyanins decreased significantly at pH above 5.0. Janna *et al.* (*27*) reported that at pH=0.5–3.0 anthocyanins of all *Tibouchina semidecandra* L. flowers at early development stages were stable, but at higher pH values the colour of the extracts faded.

The effect of temperature on the stability of anthocyanins

As shown in Fig. 3, the effect of low temperatures (≤60 °C) on the stability of anthocyanins was not significant. However, anthocyanins degraded significantly af-

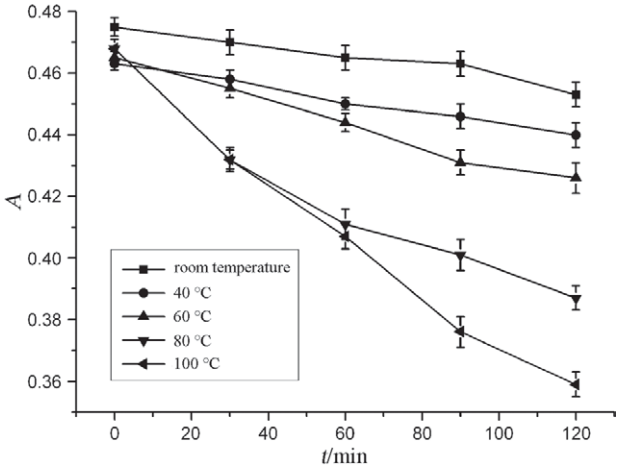


Fig. 3. Effect of temperature (room temperature, 40, 60, 80 and 100 °C) on the stability of anthocyanins. The absorbance of five extracts was determined at 535 nm at 30-minute intervals during 2 h. Each value is expressed as mean±S.D. (*N*=3)

ter exposure to higher temperatures (≥80 °C) for 2 h. The percentage of the remaining anthocyanins was only 82.7 and 76.7 % after exposure to 80 and 100 °C, respectively.

The data showed that anthocyanins were sensitive to high temperature (≥80 °C). Thus, high temperature and long heating should be avoided in the processing, storage and usage of anthocyanins. This result is in agreement with Lo Scalzo *et al.* (*28*), who reported that thermal treatments drastically reduced total cauliflower anthocyanin content. Cevallos-Casals and Cisneros-Zevallos (*21*), Kırca *et al.* (*26*), and Janna *et al.* (*27*) also reported that the increase of temperature led to the increase of anthocyanin degradation.

The effect of light on the stability of anthocyanins

As presented in Fig. 4, light greatly affected the stability of anthocyanins. The significance of the absorbance decrease was not obvious in the dark room. There was little difference in the absorbance of anthocyanins in the samples exposed to natural light indoors for different periods of time, with 86.8 % remaining after 8 days of exposure. However, the anthocyanins exposed to natural light outdoors degraded quickly and only 51.3 % remained after 8 days. Hence, strong direct light should be avoided in the processing, storage and usage of anthocyanins. They are best stored in the dark. This result was supported by Janna *et al.* (*27*), who suggested that light significantly decreased *Tibouchina semidecandra* L. anthocyanin content and led to the loss of their characteristic purplish colour. Ochoa *et al.* (*29*) also found the same effect of light on anthocyanins in raspberry, sweet (*Prunus avium*) and sour (*Prunus cerasus*) cherries.

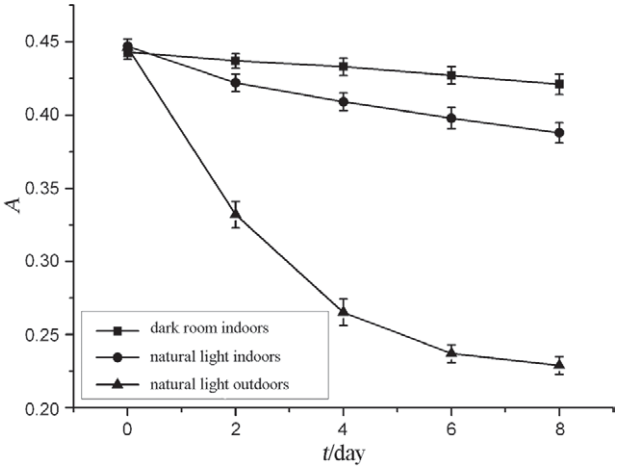


Fig. 4. Effect of light (natural light outdoors, natural light indoors and dark room) on the stability of anthocyanins. The absorbance of three same anthocyanin water solutions under different light was determined at 535 nm on the 2nd, 4th, 6th and 8th day. Each value is expressed as mean±S.D. (*N*=3)

The effect of the oxidizing and reducing agents on the stability of anthocyanins

Fig. 5 indicates that significant differences in the absorbance decrease were found in the presence of the oxidant (H_2O_2). The oxidant had a great influence on the stability of anthocyanins and caused their rapid degradation. Significant differences in the absorbance decrease of the samples in the presence of the reductant (Na_2SO_3), demonstrating that the anthocyanins had degraded rapidly, are shown in Fig. 6.

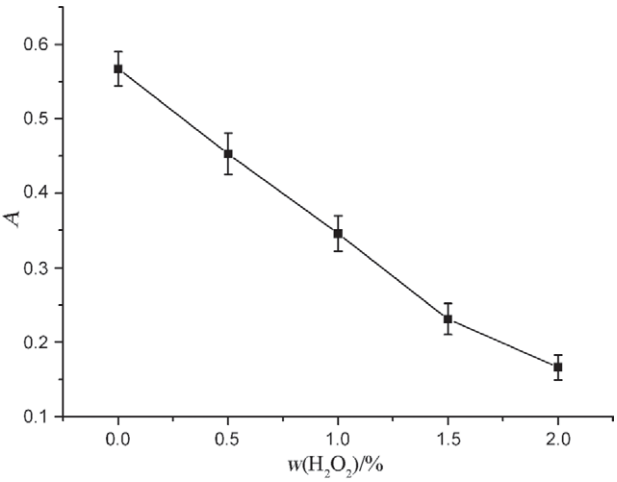


Fig. 5. Effect of H_2O_2 (0, 0.5, 1.0, 1.5 and 2.0 %) on the stability of anthocyanins. The absorbance of each sample was determined at 535 nm after one-hour exposure to room temperature. Each value is expressed as mean±S.D. (*N*=3)

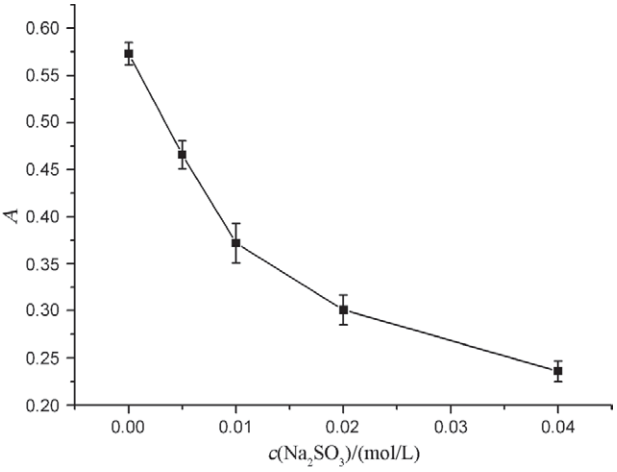


Fig. 6. Effect of Na_2SO_3 (0.005, 0.010, 0.020 and 0.040 mol/L) on the stability of anthocyanins. The absorbance of each sample was determined at 535 nm after one-hour exposure to room temperature. Each value is expressed as mean±S.D. (*N*=3)

The oxidant (H_2O_2) and reductant (Na_2SO_3) had a significant effect on the stability of anthocyanins, so their use should be avoided.

The influence of common food additives on the stability of anthocyanins

NaCl had no influence on the stability of anthocyanins (Fig. 7). The absorbance of the samples was not affected by the increase of the concentration of NaCl. Fig. 8 suggests that anthocyanins were stable in the sucrose solution and the concentration of sucrose had no effect on the absorbance of the samples after one-day expo-

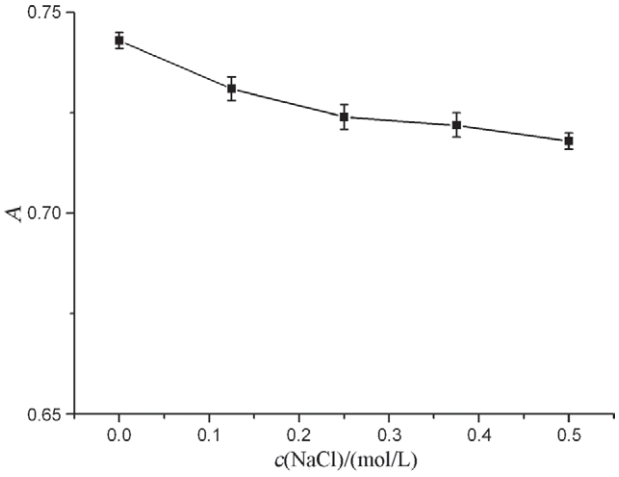


Fig. 7. Effect of NaCl (0, 0.125, 0.250, 0.375 and 0.500 mol/L) on the stability of anthocyanins. The absorbance of each sample was determined at 535 nm after one-day exposure to room temperature. Each value is expressed as mean±S.D. (*N*=3)

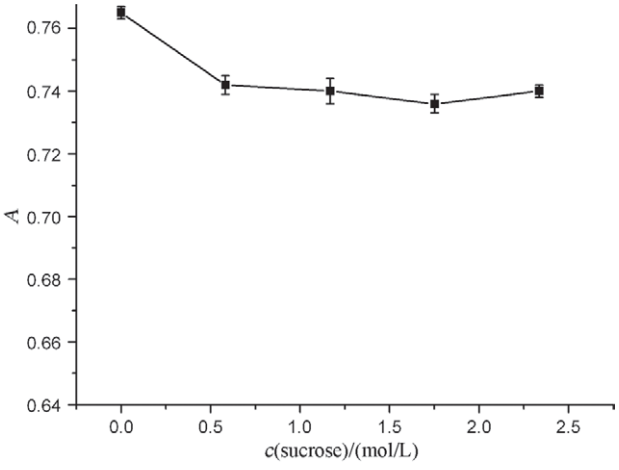


Fig. 8. Effect of sucrose (0, 0.584, 1.168, 1.752 and 2.336 mol/L) on the stability of anthocyanins. The absorbance of each sample was determined at 535 nm after one-day exposure to room temperature. Each value is expressed as mean±S.D. (*N*=3)

sure. Fig. 9 shows that preservative (sodium benzoate) did not affect the absorbance of the samples and anthocyanins were stable in sodium benzoate solution.

It is demonstrated in this study that anthocyanins were stable in the presence of common food additives. Therefore, they can be used without risk of deterioration with common food additives in food systems. This is consistent with Rizzolo *et al*. (*11*), who suggested that the addition of sugar had no effect on the anthocyanin content. Conversely, Rubinskiene *et al*. (*30*) found that fructose had negative influence on the stability of black currant anthocyanins. Sugars and salts had a negative effect on the stability of anthocyanins from acerola and açai (*31*). This might relate to the different anthocyanins found in blueberry compared to black currant, acerola and açai. Hubbermann *et al*. (*25*) were able to show that 1 to 5 % NaCl had destabilizing effect, and all sugars and sorbitol had either no effect or a stabilizing effect on

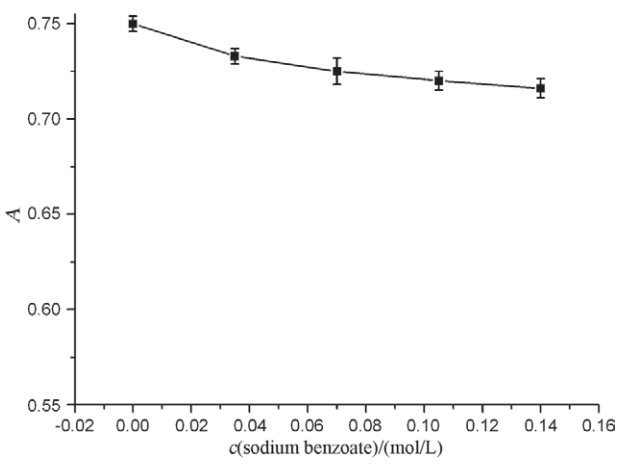


Fig. 9. Effect of sodium benzoate (0, 0.035, 0.070, 0.105 and 0.140 mol/L) on the stability of anthocyanins. The absorbance of each sample was determined at 535 nm after one-day exposure to room temperature. Each value is expressed as mean±S.D. (*N*=3)

the colour of black currant and elderberry concentrates. This NaCl range was to some extent above that presented in this paper (0.125–0.500 mol/L). Therefore, it seems that lower concentrations (≤0.500 mol/L) of NaCl did not affect the stability of anthocyanins.

The stability of anthocyanins strongly depends on their structure and chemical composition of the anthocyanin source (*25,32*). In different pH solutions, one anthocyanin can be transformed to another (*25*). Therefore, the stability of anthocyanins changes as a function of pH. The differences in stability between different fruits and plants could be due to the differences in the composition of the anthocyanins in them.

Antioxidant activity protecting ECV-304 cell against H_2O_2 damage

Fig. 10 shows that the absorbance measured by MTT method of the cells pretreated with 15, 25, 50 and 100

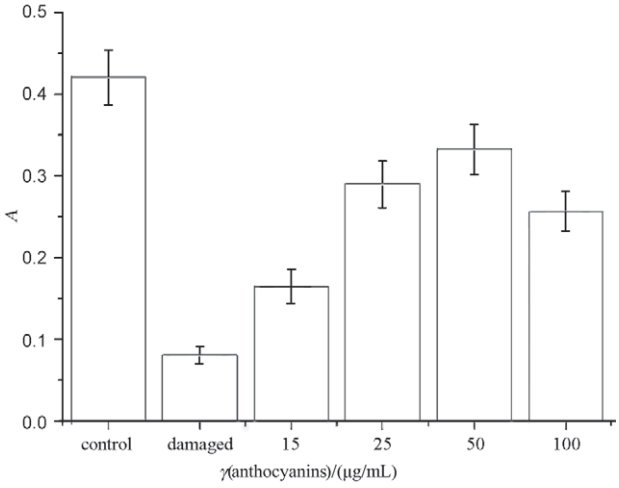


Fig. 10. Protection of anthocyanins at different concentrations (15, 25, 50 and 100 μg/mL) against oxidative damage induced by H_2O_2. The absorbance was determined at 570 nm with MTT method. Each value is expressed as mean±S.D. (*N*=3)

μg/mL of anthocyanins was significantly ($p<0.01$) higher than that of the damaged cells (treated with H_2O_2). This suggests that anthocyanins at these concentrations protect the cells. The survival rate (absorbance of samples/absorbance of the control) of the four treated cells (with 15, 25, 50 and 100 μg/mL of anthocyanins) rose to 38.67, 68.97, 79.00 and 61.10 %, respectively from 19.09 % of the damaged cells. This may be due to the fact that anthocyanins are strong antioxidants. They can interact with biological systems through many potential pathways, such as oxygen radical absorbance, chelating of the metal ions, and free radical scavenging (*33*). Therefore, they can weaken oxidative and inflammatory stress and alleviate some of the deleterious effects related to reactive oxygen species production (*34*).

Morphological changes of ECV-304 cells were observed with optical microscope. As presented in Fig. 11a, the control cells were flat, long fusiform, polygonal or elliptical mosaic, uniform in size, with a regular shape, plump, and had clear and continuous borders. The cells showed no overlapping, and the nucleus was located in the center of the cells. The cells treated with H_2O_2 were elongated, some cells contracted, became rounded and smaller and the intercellular space was enlarged; most cells were disrupted, deciduous, but still had clear cellular borders (Fig. 11b). The damage to the cells treated with 15, 25, 50 and 100 μg/mL of anthocyanins was less (Fig. 11c–f) than that to the H_2O_2-treated cells. Cell morphology generally improved. This suggests that anthocyanins can protect the cells against H_2O_2 damage.

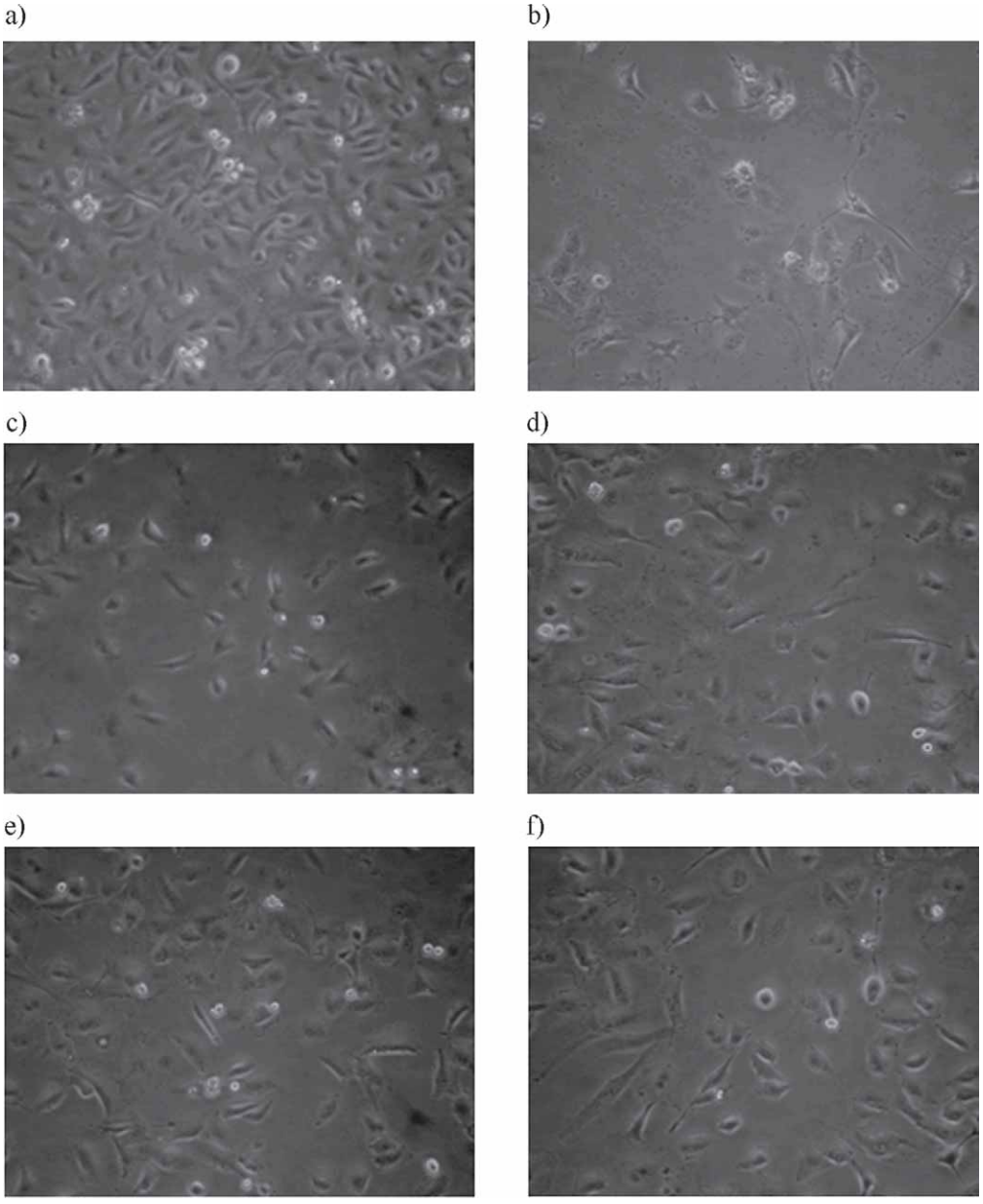


Fig. 11. Cell protection (morphological changes of ECV-304 cells) by anthocyanins against oxidative damage induced by H_2O_2. Each value is expressed as mean±S.D. (N=3). a) normal control cells, b) cells damaged by 1 mmol/L of H_2O_2, c) cells treated with 15 μg/mL of anthocyanins and 1 mmol/L of H_2O_2, d) cells treated with 25 μg/mL of anthocyanins and 1 mmol/L of H_2O_2, e) cells treated with 50 μg/mL of anthocyanins and 1 mmol/L of H_2O_2, f) cells treated with 100 μg/mL of anthocyanins and 1 mmol/L of H_2O_2

Polyphenols have also been found to possess prooxidative effects at high concentrations, such as cherry liqueur extract (*35*), quercetin (*36*) and green tea polyphenols (*37*). In this study, we found that the best concentration of anthocyanins to protect the ECV-304 cells from H_2O_2 damage is 25–50 μg/mL. At 100 μg/mL, the survival rate of cells is 61.10 %, lower ($p<0.05$) than that of 79.00 % at 50 μg/mL and 68.97 % at 25 μg/mL. Further studies are needed to determine the prooxidative activity of anthocyanins. Hence, they could protect cells from oxidative deterioration and could be considered as a potential health diet and functional food at 25–50 μg/mL to prevent and cure diseases related to oxidative activity. However, further pharmacology and toxicity studies of anthocyanins should be carried out *in vivo*.

Conclusions

In conclusion, results from this study clearly indicate that anthocyanins from blueberry were stable at low pH (≤5.0), and not affected by different concentrations of NaCl, sucrose and preservatives, but were sensitive to alkaline environment, high temperature, light, oxidants and reductants. Anthocyanins could protect ECV-304 cells against antioxidant damage induced by H_2O_2. Moreover, the results showed that high temperatures (>80 °C), long heating and direct strong light, as well as oxidizing and reducing agents should be avoided in the processing, storage and utilization of anthocyanins. Common food additives could be used with anthocyanins without risk. They could be considered as potential colorant in acidic foods. Furthermore, anthocyanins could be used as a health food to control diseases arising from oxidative activity. Further research is necessary to isolate and characterize the monomeric anthocyanins to elucidate their stability and the existence of possible synergisms enhancing their stability.

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