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Extraction of Anthocyanins from Black Grape By-Products and Improving Their Stability Using Cobalt(II) Complexation

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ABSTRACT: This study was conducted to investigate the effect of cobalt complexation on the spectral properties of anthocyanins (AC) extracted from black grape pomace (Black Magic) and the effect of complexation on the pH stability of AC during storage. Initially, cobalt acetate tetrahydrate aqueous solution was complexed with AC crude extract and diluted separately in buffer solutions with different pH (3.5, 4.5, 5.5, and 6.5). Afterward, spectral changes were determined spectrophotometrically. pH stability was investigated using the same buffer solutions and stored for 7 days in the dark at room temperature, and the absorbance of each solution was measured daily using a spectrophotometer. Results indicated that complexation caused similar hypsochromic and hyperchromic shifts in λ_{max} at all pH values. With regard to pH stability, the degradation of complexed AC followed first-order reaction kinetics causing half-lives to increase up to 80-fold as compared with noncomplexed AC, which was due to the sharp decrease in K (per day), indicating an improved pH stability as compared with noncomplexed AC. Therefore, Co(II) could be used in the stabilization of grape AC for the coloration of a wide range of foods and food products at near-neutral pH environments considering the health benefits of grape AC and the maximum nontoxic dose of Co(II) salt.

Keywords: anthocyanins, cobalt(II), grapes, *Vitis*

INTRODUCTION

Anthocyanins (AC), a group of water-soluble pigments, are abundant in nature, and they provide red, purple, and blue colors to fruits, vegetables, legumes, flowers, and other colorful plants because of the conjugated bonds in their chemical structure (Grotewold, 2006). They are a major subclass of the flavonoid family (Landi et al., 2015). Hence, they acquire antioxidative, anti-inflammatory, and anticarcinogenic properties (Khoo et al., 2017). However, they have limited stability toward several factors such as pH, temperature, light, oxygen, enzymes, and copigments (Amr and Al-Tamimi, 2007; Turturică et al., 2015).

AC color changes in response to pH variations (Dangles and Fenger, 2018). In highly acidic media, AC assume a red color (flavylium cation). However, upon increasing the pH (4.0∼5.5), they become colorless (carbinol pseudobase) probably because of the deportation of the flavylium cation. At neutral pH, a bluish-purple color (quinoidal base) starts to appear, whereas at pH above 8, a yellow color (chalcone) persists (Wrolstad, 2004; Nave et al., 2010).

Apart from copigmentation and encapsulation (Guldiken et al., 2021), metal complexation is a common approach for the stabilization of AC color (Gençdağ et al., 2022). Transition metals were used to form complexes with AC (Estévez et al., 2021), which have chemical structures with *ortho* dihydroxyl groups on the B ring such as cyanidin, petunidin, and delphinidin (Cavalcanti et al., 2011), thereby causing bathochromic and hyperchromic shifts in the absorbance of maximum wavelength (Sigurdson et al., 2016). Metal ions, such as Fe^{2+}, Fe^{3+} (Tachibana et al., 2014), Al^{3+} (Sigurdson and Giusti 2014; Sigurdson, 2016), and Zn^{2+} (Zhao et al., 2020), have been used in the complexation of AC.

Red and black grapes, *Vitis vinifera*, are rich sources of AC, which provide the distinctive color to wine and grape juice (Georgiev et al., 2014). Several wineries and grape

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juice production plants worldwide use black grapes as a starting material for their wine and juice production. A sizable amount of AC-rich waste in the form of peels, seeds, and stems is generated from their operations (Iannone et al., 2021). However, grape AC are among the least stable ones (Westfall, 2015). Given that malvidin is the most dominant, the lack of an *ortho* dihydroxyl group on the B ring limits metal complexation (Rustioni, 2015).

To the best of our knowledge, data supporting the use of metals, particularly Co(II), in the complexation of grape AC at near-neutral pH are not available at present. Therefore, this study aims to determine the extraction yield of black grape pomace (Black Magic) using two solvent systems at varying water-to-alcohol ratios, study the spectral changes associated with the complexation of Co (II) with grape AC, and determine the AC degradation kinetic parameters at different pH values before and after complexation during 1 week of storage.

MATERIALS AND METHODS

Plant material and chemicals

Black grapes (Black Magic) were purchased from a local grocery in Amman (Jordan) and stored in a refrigerator until usage. Grape berries were manually removed from stems and seeds and stored frozen in plastic bags until extraction. All chemicals were purchased form a local supplier in Amman (Jordan).

Extraction of AC from black grape pomace

In brief, frozen black grape pomace was thawed at room temperature and dried in an oven at 50°C to a constant weight. Dried pomace was ground using a coffee grinder and extracted with acidified aqueous methanol and aqueous ethanol (0.01% HCl) at varying alcohol-to-water ratios (50, 60, 70, 80, 90, and 100; v:v). The extracts were vacuum filtered, and the process was repeated until the extract became colorless. The solvent was evaporated under reduced pressure using a rotary evaporator (Buchi, Flawil, Switzerland), and the resulting concentrate was reconstituted with deionized water, placed in a screw-capped flask after flushing with nitrogen, and stored in a freezer until purification.

Purification of AC

AC were purified through loading onto Diaion HP-20 macroporous resin (Sigma-Aldrich Co., St. Louis, MO, USA) partially filled in a glass column. After loading of AC and washing several times with deionized water to remove sugar and organic acids, acidified methanol and ethanol (100%) were used separately to eluate AC. Afterward, the solvent was evaporated using a rotary evaporator, and the subsequent concentrate was diluted with deionized water, centrifuged at 10,000 rpm for 10 min, flushed with nitrogen, and stored in a freezer until further usage.

Quantification of monomeric AC

AC yield in black grape pomace was determined before purification using the pH differential method described by Giusti and Worlstad (2001) using the following equation:

$$\text{Total AC (mg/L)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon}$$

$$A = (A_{520\ nm} - A_{700\ nm})\ \text{pH 1.0} - (A_{520\ nm} - A_{700\ nm})\ \text{pH 4.5}$$

where A is absorbance, MW is the molecular weight of cyanidin-3-glucoside (449.2 g/mol), DF is the dilution factor, 10^3 is the factor used to convert g to mg, and ε is the molar extinction coefficient of cyanidin-3-glucoside (26,900 L/mol/cm).

The concentration of AC was expressed as cyanidin-3-glucoside equivalent in mg/L.

Complexation of AC with Co(II) ions

In brief, 1 mL of 30 mg/L AC was mixed with 1 mL of 25 g/L cobalt acetate tetrahydrate aqueous solution and allowed to stand for 30 min in the dark. Afterward, the mixture was diluted separately with 8 mL of citrate-phosphate buffer solution (0.1 M citric acid monohydrate+ 0.2 M disodium hydrogen phosphate) at different pH values of 3.5, 4.5, 5.5, and 6.5. Changes in spectral properties [lambda max (λ_{max}) and relative absorbance] were recorded for each pH (for AC and AC-cobalt complex) using a ultraviolet-visible spectrophotometer (Varian Medical Systems Australasia, Belrose, NSW, Australia).

Stability to pH

AC [either alone or complexed with Co(II)] were tested for stability against four pH values of 3.5, 4.5, 5.5, and 6.5 (prepared from the aforementioned citrate-phosphate buffer solution) for 7 days in the dark at room temperature. The absorbance was read on day zero, 1, 2, 3, 4, 5, 6, and 7, and the results were expressed as AC retention (AR) based on the following equation:

$$AR = \frac{\text{Absorbance of AC at any given time}}{\text{Absorbance of AC at zero time}}$$

Cytotoxicity evaluation of AC-Co(II) complexes

Initially, five AC-Co(II) complexes were prepared by mixing AC (at a given concentration) with different cobalt acetate tetrahydrate solutions and concentrations. The final AC concentration was 170 ppm in all complexes

(fixed), whereas the final concentration of cobalt salt was 85, 8.5, 0.85, 0.085, and 0.0085 ppm in different complexes (variable).

The toxicity of AC-Co(II) complexes was assessed in accordance with the method of Tarawneh et al. (2021) with some modifications using human fibroblasts as a normal cell line. Cells were cultured in Dulbecco's modified Eagle's medium (EuroClone S.p.A, Pero, Italy) supplemented with 1% penicillin-streptomycin (EuroClone S.p.A), 1% L-glutamine, and 10% fetal bovine serum. Cells were incubated in a 37°C incubator under 5% CO_2 atmosphere until they become confluent.

Cells were seeded in 96-well plates (10,000 cells/well), treated with the aforementioned complexes, and incubated for 72 h to screen for cellular toxicity. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium was performed according to the manufacturer's protocol to detect the metabolic action of living cells using the CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega Corp., Madison, WI, USA). Then, the absorbance was recorded at 570 nm using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA). Cell viability was calculated using the following equation:

$$\text{Cell viability (\%)} = \frac{\text{Absorbance of treated cells}}{\text{Mean absorbance of control cells}} \times 100$$

Statistical analysis

Total AC data were analyzed by analysis of variance using Statistical Package for Social Sciences (SPSS version 22.0, IBM Corp., Armonk, NY, USA). Differences among means were determined by the least significant difference test and considered significant at $P<0.05$.

RESULTS AND DISCUSSION

Extraction yields

Total AC (mg/L) in black grape pomace are presented in Fig. 1. Pure methanol was significantly ($P<0.05$) more effective than pure ethanol in extracting AC, whereas 50% ethanol obtained the highest yield among the aqueous solutions. Notably, the higher the methanol concentration is, the higher the AC content is. On the contrary, a nonconsistent pattern was observed with the decrease of ethanol concentration, although 50% ethanol yielded higher content than pure ethanol.

Solvent extraction is frequently used in the isolation of AC from their natural sources (Zou et al., 2011). AC are polar in nature because of the presence of hydroxyl, carbonyl, and methoxy functional groups as well as sugar

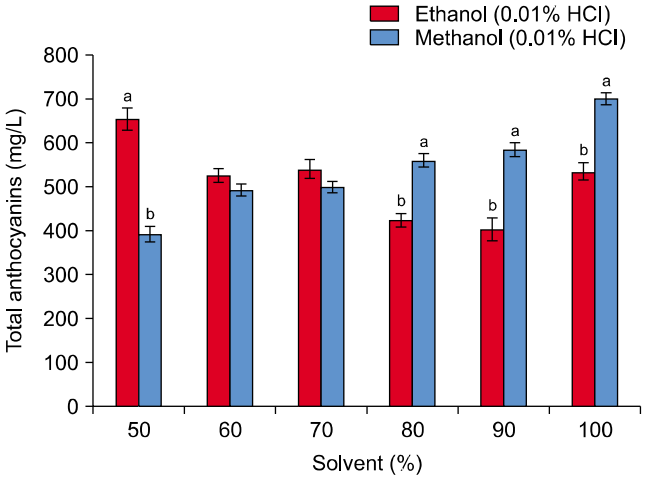

Fig. 1. Total anthocyanins (mg/L) in black grape pomace. Values are presented as mean±SD (n=3). Significant differences ($P<0.05$) among different solvents at the same concentration are indicated in letters (a,b).

moiety in their chemical structure (Bueno et al., 2012). Therefore, polar organic solvents, such as methanol and ethanol and to a lesser extent acetone and acetonitrile, are often used in the extraction of AC from their natural matrices. These solvents can rupture the tissue compartment in which AC are located, thereby releasing and stabilizing them (Naczk and Shahidi, 2006). Moreover, acidification of the solvent system is important because it facilitates the solubilization of AC from plant tissues, thereby increasing the extraction yield and stabilizing the recovered AC by maintaining their flavylium cation form (Paun et al., 2017). Furthermore, the acid : solvent ratio plays a role in the extraction yield of AC; increasing the acid (HCl) : solvent ratio led to a higher AC yield from *Ranunculus asiaticus* (Amr and Al-Tamimi, 2007) and radish (Giusti and Wrolstad, 2001), which might be due to the high molar absorptivity of AC (Giusti and Wrolstad, 2001). In this study, 0.01% HCl was used because a higher ratio may subject the pigment to partial hydrolysis (Welch et al., 2008).

Similar to our results, Paun et al. (2022) and Metivier et al. (1980) found that methanol was superior to ethanol (at the same concentration) in extracting AC from grape pomace. However, the efficiency of different solvents varies with the plant material from which AC are extracted (Paun et al., 2017). For example, water was found to be more efficient than methanol and ethanol in recovering AC from purple corn (Jing et al., 2007). Meanwhile, ethanol was not as effective as methanol in extracting AC from *R. asiaticus* (Amr and Al-Tamimi, 2007).

Effect of Co(II) addition on the spectral properties of AC at different pH values

Changes in λ_{max} and relative absorbance are listed in Table 1 and Fig. 2, which show that the complexation of

AC with cobalt cations caused hyperchromic and hypsochromic effects on the maximum absorbance at all pH values; the blue shift was supported by the formation of a pinkish-red color instead of a bluish-purple one.

Regarding AC without complexation, λ_{max} was in the range of 517~524, representing typical absorption peaks. Absorbance was the highest at pH 3.5, after which it began to decrease with the increase of pH; the lowest absorbance was recorded at pH 5.5. However, absorbance at pH 6.5 was higher than that at pH 4.5 and 5.5 but lower than that at 3.5.

When complexed with Co(II), the pattern was different; absorbance was the highest at pH 6.5 followed by pH 5.5 and pH 3.5, and the lowest at pH 4.5. Notably, all samples had similar λ_{max} range (within a 5-nm difference).

Cyanidin 3-*O*-glucoside and delphinidin-3-glucoside were complexed with ferrous ions at pH 6; hyperchromic (increased intensity) and bathochromic shifts in the maximum absorbance were observed (Tachibana et al., 2014). In another study, Al^{3+} at varying concentrations was used in the complexation of AC extracted from eggplant (a delphinidin-rich source). After complexation and testing the spectral properties of AC at varying pH values (pH 3~6), all samples exhibited a hyperchromic shift, with complexed solutions at higher pH values having lower intensity than those at lower pH values. However, samples at lower pH values exhibited a bathochromic shift, whereas those at pH 6 showed a hypsochromic shift, particularly when high salt concentration was used (Sigurdson, 2016). Therefore, the effect of metal chelation on the color stability of AC in response to pH depends primarily on the metal cation used and the type of anthocyanidin. Malvidin is the major anthocyanidin found in grapes representing 60~70% of the total AC (Rustioni, 2015). In this study, considering that malvidins cannot chelate metals (Boulton, 2001; Dixon et al., 2005), minor anthocyanidins in the form of cyanidin, delphinidin, and petunidin primarily contribute to metal chelation. For example, Rustioni (2015) suggested that non-*ortho* dihydroxyl groups and copigments could have a considerable effect on grape AC

Table 1. Effect of Co(II) complexation on the spectral properties of AC at different pH values

pH	λ_{max} of AC	Relative absorbance % of AC	λ_{max} of AC-Co(II)	Relative absorbance % of AC-Co(II)
3.5	517	100.00	512	100.00
4.5	519	67.34	513	99.15
5.5	524	62.31	516	101.14
6.5	524	86.43	516	109.40

AC, anthocyanins; λ_{max}, wavelength of maximum absorbance.

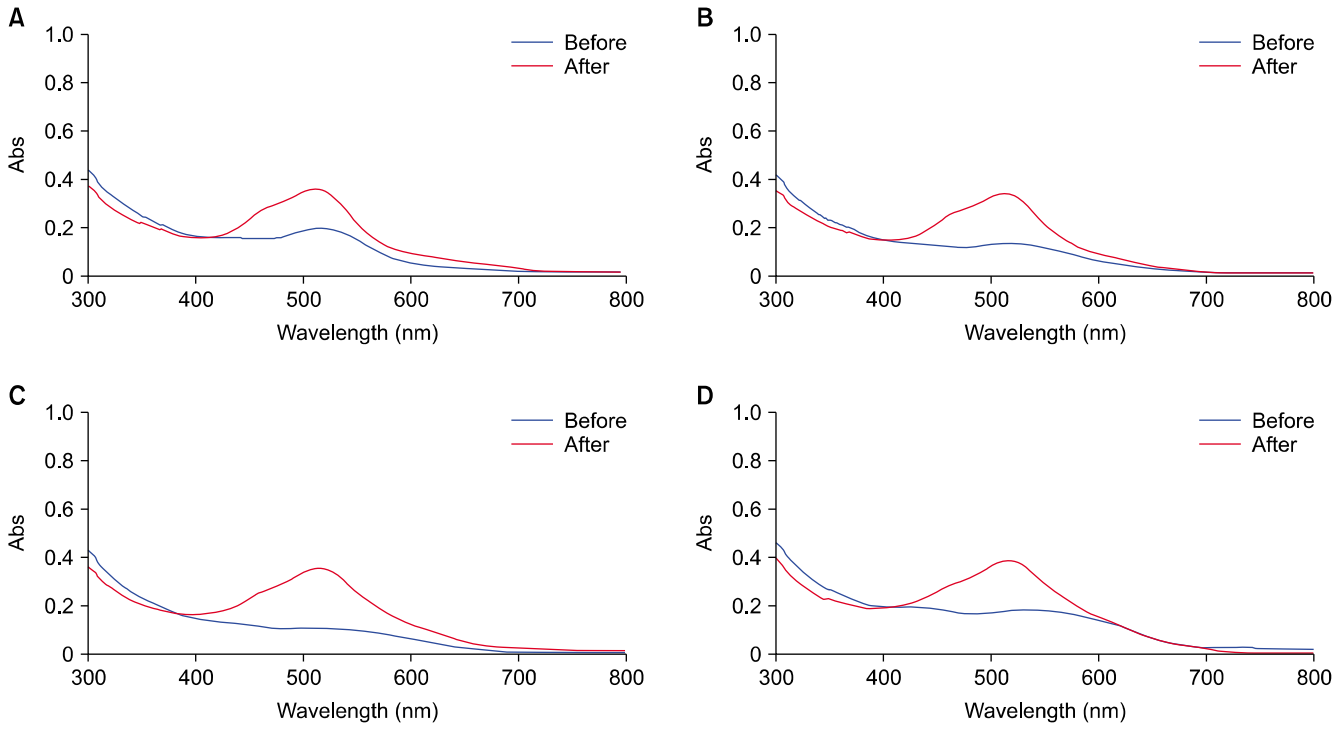

Fig. 2. Absorption spectra of anthocyanins before and after complexation with Co(II) at pH 3.5 (A), pH 4.5 (B), pH 5.5 (C), and pH 6.5 (D). The peak of each band represents the wavelength of the maximum absorbance. Abs, absorbance measured by a spectrophotometer.

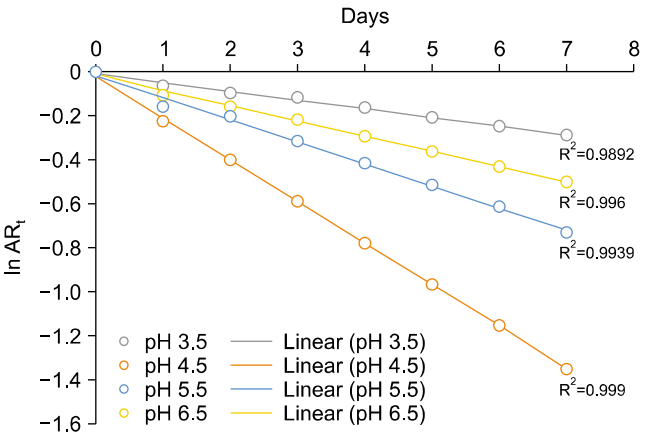

Fig. 3. Degradation of anthocyanins (AC) during storage at different pH values. Each regression line represents the relationship between the dependent variable (ln AR_t) and independent variable (storage time in days) at a given pH value. ln AR_t, natural logarithm of AC retention after a certain storage time; R^2, coefficient of determination (a value of 1.0 indicates a perfect fit).

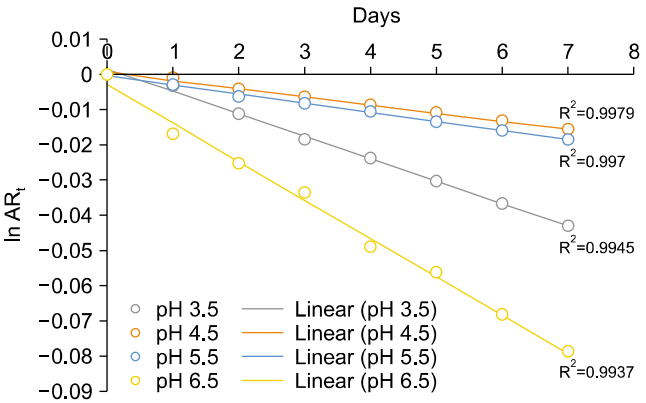

Fig. 4. Degradation of the anthocyanins (AC)-Co(II) complex during storage at different pH values. Each regression line represents the relationship between the dependent variable (ln AR_t) and independent variable (storage time in days) at a given pH value. ln AR_t, natural logarithm of the AC-Co(II) complex retention after a certain storage time; R^2, coefficient of determination (a value of 1.0 indicates a perfect fit).

added with Cu(II) (Sangiovese and Cabernet Sauvignon) and found that higher concentrations of Cu(II) resulted in hyperchromic and slight hypsochromic shifts, which were consistent with our results.

Degradation kinetics of AC during storage at different pH values

Fig. 3 and 4 show that the degradation of AC (without and with complexation) followed the first-order reaction kinetics when the natural logarithm of AR was plotted against time in days.

The reaction rate constants (K) and half-lives (t½) of AC before and after complexation (Table 2) were determined using the following equations:

$$\ln AR_t = -K \times t + \ln AR_0$$

Table 2. Kinetic parameters for AC degradation before and after Co(II) complexation during storage at different pH values (unit: per day)

pH	K	t½
3.5		
AC	3.99×10^{-2}	17.37
AC-Co(II)	6.40×10^{-3}	108.28
4.5		
AC	1.899×10^{-1}	3.65
AC-Co(II)	2.30×10^{-3}	301.30
5.5		
AC	1.01×10^{-1}	6.89
AC-Co(II)	2.6×10^{-3}	266.54
6.5		
AC	6.92×10^{-2}	10.02
AC-Co(II)	1.09×10^{-2}	63.58

AC, anthocyanins; K, reaction rate constant; t½, half-lives.

where AR_0 is the initial AR and AR_t is the AR after t (day) at a given pH value.

$$t_{1/2} = \frac{0.693}{K}$$

In general, Co(II) complexation caused a decrease in K at each pH tested, thereby increasing the half-lives. The half-life of AC was the highest at pH 3.5, and it increased by at least six times (from 17.37 to 108.28 days) after complexation. Surprisingly, AC before complexation had the lowest half-life at pH 4.5, but it increased about 80 times after complexation (from 3.65 to 301.30 days). On the contrary, complexation increased the half-life of AC by at least 30 times (from 6.89 to 266.54 days) at pH 5.5. The half-life of AC stored at pH 6.5 was 10 days prior to complexation, which increased the half-life of AC by six times.

In general, the pH stability of AC after 7 days of storage decreased in the following order: pH 3.5> 6.5> 5.5> 4.5. In the case of complexation, stability decreased in the following order: pH 4.5> 5.5> 3.5> 6.5.

It is well-known that AC are more stable in highly acidic environments, and they become less stable as the pH increases (Giusti and Wrolstad, 2001). However, this finding does not apply to metal-complexed AC. For example, Mustika and Marpaung (2019) found that AC extracted from Buni fruit and complexed with either Al^{3+} or Fe^{3+} showed lower stability than AC alone, although the metal complexes showed maximum stability at pH 6 when they were tested at several pH values (3~8). On the contrary, the half-life of petunidin derivatives was higher than that of petunidin alone when complexed with trivalent Al and Fe, particularly at pH 8, indicating enhanced stability over the storage period (Tang and Giusti, 2020). Therefore, the pH stability of AC-metal complexes depends primarily on the metal cation used (high- or low-

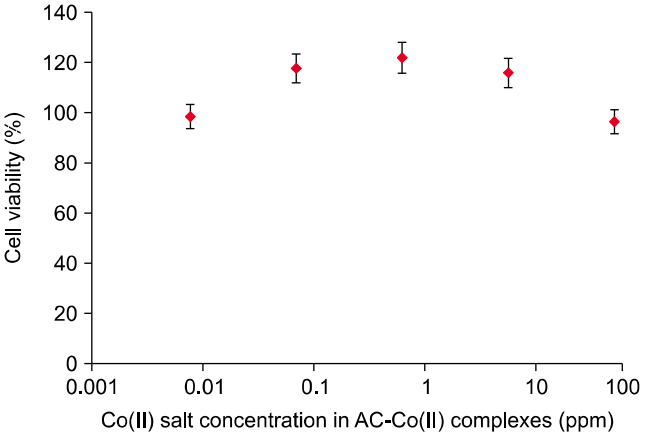

Fig. 5. Toxicity of anthocyanins (AC)-Co(II) complexes against human fibroblasts as the cell line. Concentration of AC is fixed in all samples, whereas concentration of cobalt acetate tetrahydrate is varied. Values are presented as mean±SD (n=3). Values higher than 80% are considered not toxic. Cell viability (%) = (absorbance of treated cells / mean absorbance of control cells) ×100.

spin configuration) and the source of anthocyanidins. The enhanced stability of the AC-metal complexes in low-acid to near-neutral media could be explained by the fact that the flavylium cation loses its charge at nearly neutral pH (quinoidal base) environments, which makes it easier for metal cations to chelate AC (Mustika and Marpaung, 2019).

Cytotoxicity of AC-Co(II) complexes

The effect of cobalt salt concentration in AC-Co(II) complexes on cell viability (%) is presented in Fig. 5, which shows that cell viabilities were higher than 96%. Considering that cell viability is a measure of toxicity (inverse relationship), concentrations with cell viabilities higher than 80% are considered safe (Horák et al., 2017). Therefore, no toxicity was found at the given concentration range (0.0085～85 ppm).

Grape AC were successfully complexed with cobalt ions, although minor anthocyanidins participated only in the complexation process. This finding was confirmed by the hypsochromic effect upon the addition of the buffer solutions. However, this blue shift was advantageous, that is, a pinkish-red color appeared instead of a bluish-purple color, which makes it favorable to be added into a wide range of food products at nearly neutral pH considering the highest nontoxic cobalt salt concentration found in the present study.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Concept and design: AA. Analysis and interpretation: AA, SJ, IH, HH. Data collection: SJ, MZ. Writing the article: SJ. Critical revision of the article: AA, LH, HH. Final approval of the article: all authors. Statistical analysis: MZ. Obtained funding: AA, MS. Overall responsibility: HA, SJ, AA, IH.

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