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Optimisation of Anthocyanin Extraction from Purple Pitaya and Verification of Antioxidant Properties, Antiproliferative Activity and Macrophage Proliferation Activity

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Abstract

To separate and purify anthocyanins from purple pitaya (*H. polyrhizus*) with SP825 resin, the adsorption properties of the SP825 resin were evaluated by static and dynamic experiments with respect to the following parameters: pH, temperature, dilution multiple of the sample solution and concentration of the ethanol eluting agent. The data from the static experiments were fitted to the pseudo-second-order kinetics model ($R^2 = 0.9919$) and Freundlich isotherm model ($R^2 = 0.9672$). A total of 658 mg of anthocyanins were contained in 1000 g of *H. polyrhizus* fresh fruits (both peel and pulp), which could be made into 1.5 g of anthocyanin-rich extract from *H. polyrhizus* (AEHP) containing 520 mg of anthocyanins. Overall, 79% of the anthocyanins were extracted. The AEHP showed strong DPPH radical scavenging activity, ABTS radical scavenging activity, hydroxyl radical scavenging activity, reducing power, ferrous metal ion chelating ability and SOD-like activity. Moreover, the AEHP inhibited the proliferation of the human colorectal cancer cell line DLD-1 and accelerated the growth of macrophage cells. Finally, the AEHP was proven to have a high tinctorial power and physiological and health functions.

Keywords: purple pitaya, anthocyanins, resin, antioxidant properties, antiproliferative activity, macrophage Proliferation activity

1. Introduction

Edible colorants include natural and synthetic colorants. Because synthetic colorants have toxic effects on humans, natural colorants have drawn more attention (Chou et al., 2007). Based on the chemical structure, natural colorants have been classified into tetrapyrroles, carotenoids, polyphenolic compounds, and alkaloids. As a type of phenolic compound, anthocyanins are plant pigments that are widely distributed in nature (Benoit, 2004; Wu & Prior, 2005; Yang & Zhai, 2010). Additionally, anthocyanins are considered to be food colorants and health food materials (Smith et al., 2000; Wang et al., 2000; Cooke et al., 2005; Pergola et al., 2006).

The pulp colour in *Hylocereus* fruits varies from white to red and purple. *Hylocereus cacti* (*H. cacti*) have red pulp, and *Hylocereus polyrhizus* (*H. polyrhizus*) have purple pulp. *Hylocereus* fruits might have a potential tinctorial power, and some researchers have studied the antioxidant and antiproliferative activities of *H. cacti* (Wu et al., 2006). The results revealed that the acetone extracts of *H. cacti* appeared to have certain DPPH and ABTS radical scavenging activities and to inhibit the growth of B16F10 melanoma cells. Moreover, the anticancer activities have been widely investigated in anthocyanin-rich extracts from the berry (Hogan et al., 2010). However, there is little information on the antioxidant and antiproliferative and macrophage proliferation properties of the AEHP with resin (Wang et al., 2008). Therefore, an efficient extraction method was warranted for testing. Using this method, we obtained an AEHP that had a high tinctorial power. Finally, the physiological and health functions of the AEHP were also evaluated by antioxidant, antiproliferative and macrophage proliferation activity assays.

2. Materials and Methods

2.1 Sample Preparation

H. polyrhizus fruits (both peel and pulp) were collected in the summer from Yunnan Province, China. First, the fruits were juiced and stored under -20°C for 2 months. Next, they were thawed at 25°C, stirred and divided into 50 g portions. The samples were diluted, and the pH values were adjusted before they were filtered through 300 mesh gauze. Finally, diatomite powders were added at a ratio of 1.7 gram: 100 millilitres (diatomite powder: sample juice), and the mixtures were centrifuged for 5 min at 7000 r / min to collect the supernatant for testing.

2.2 Resin Pretreatment

The SP825 resin was soaked in 95% ethanol, adopting the wet packing method, for 24 h. The resin fully swelled, which removed the impurities, gas and floating resin debris. Then, the resin columns were washed with distilled water thoroughly (Fan et al., 2008).

2.3 Static and Dynamic Adsorption / Desorption Tests

2.3.1 Static Adsorption / Desorption Tests

First, 1 g of resin was placed in a tube with a lid. Next, the pH of the sample was adjusted from 1 to 9 with 0.1 M hydrochloric acid. After adding 15 mL of the sample, the tubes were incubated at 25 °C for 2 h. Then, the sample was scanned between 400 and 750 nm (Shimadzu UV-1800). The absorbance values of the sample were measured at the maximum absorption wavelengths (λ_{max}). The tubes without resin were the controls. Finally, the optimum pH was evaluated according to the adsorption and degradation ratios as follows:

$$Adsorption\ ratio\ (\%) = (A_{control} - A) / A_{sample} \times 100\% \quad (1)$$

$$Degradation\ ratio\ (\%) = 1 - A_{control} / A_{sample} \times 100\% \quad (2)$$

where A was the absorbance of the sample after adsorption for 2 h, A_{sample} was the initial sample absorbance, and $A_{control}$ was the absorbance of the control sample after 2 h.

A total of 1 g of resin was placed in a tube with a lid. Next, the pH of the sample was adjusted according to the static test results. After adding 15 mL of the sample, the tubes were incubated for 6 h at adsorption temperatures of 25, 30, 40, 50 and 60°C. The absorbance values of the samples were measured at λ_{max}. The tubes without resin were the controls. Finally, the optimum temperature was evaluated.

For the next test, 1 g of resin was placed in a triangular bottle with a cork. Next, the pH and temperature of the sample were adjusted according to the static test results. After adding 100 mL of the sample, the bottles were shaken for 16 h (180 r / min). The bottles without resin were the controls, and the sampling interval was 0.5 h. Then, the absorbance values were measured at λ_{max} to determine the anthocyanin concentrations in the adsorption process until equilibration. Finally, the kinetics curve was drawn according to the data.

A total of 0.1 g of resin was placed in a tube with a lid. Next, 10 mL of the sample, each containing a different concentration of anthocyanins, was added to the tube and shaken at 180 r / min for 8 h at 25°C. The tubes without resin were the controls. Then, the absorbance values were measured at λ_{max} to determine the anthocyanin concentrations in the adsorption process until equilibration. Finally, an isotherm curve was drawn according to the data.

2.3.2 Dynamic Adsorption / Desorption Tests

Based on the static tests, the dynamic tests were investigated with respect to the following parameters: pH, the dilution multiple of the sample and the concentration of the ethanol eluting agent. Adopting the wet packing method, the dynamic tests were conducted in a glass column packed with 18 g (dry height) of resin. The volume of the resin bed (1.9 cm × 13 cm) was 37 mL, and the ratio of its diameter to height was approximately 1:7. Second, the resin columns were pretreated and 50 g of sample was thawed and diluted. Then, the pH values were adjusted. Third, the samples were filtered through 300 mesh gauze, and diatomite powders were added at a ratio of 1.7 gram: 100 millilitres (diatomite powder: sample juice), and the mixtures were centrifuged. Next, the supernatant, 3 BV (resin bed volume) distilled water and 3 BV ethanol eluting agent were passed through the resin columns at 3 mL / min in that order. The collected eluted liquid was rotated and evaporated for 90 min at 40 °C and freeze-dried into a powder. Finally, the anthocyanin concentrations of the powder were determined, and the optimum conditions were determined.

2.4 The Determinations of the Total Anthocyanin and Polyphenol Contents and E

2.4.1 The Determination of the Total Anthocyanin Content

A total of 5 mg of the AEHP powder was dissolved in 5% formic acid up to a volume of 100 mL in a volumetric flask. The solution was diluted five times and scanned to obtain a λ_{max} between 500 and 550 nm. Then, the absorbance was measured at $\lambda_{max.}$ Blueberry powders were used as a standard, and its anthocyanin content was 26.2%.

$$The\ Anthocyanins\ Content\ of\ the\ Sample = (A / B)\ 26.2\% \quad (3)$$

where A was the absorbance of the sample and B was the absorbance of the standard sample.

2.4.2 The Determination of the Phenolic Content

The phenolic compounds were determined using the Folin-Ciocalteu method with some modifications (Mau, et al., 2002). The AEHP was completely dissolved in distilled water with a concentration of 2 mg/mL. The solution (0.125 mL) was mixed with distilled water (0.375 mL) and 0.5 mL of the Folin-Ciocalteu reagent. After 3 min, 0.5 mL of Na_2CO_3 (20%) was added, and the mixture was brought to a final volume of 5 mL with distilled water. After being kept in the dark for 90 min, the O.D. of the mixture was read at 725 nm.

2.4.3 The Determination of the E of the AEHP by the CU Method

The CU method is often used to represent the dyeing capability of a natural edible pigment. The CU is also known as the ratio of absorption value, i.e., the absorption value of 100 mL of a solution containing 1 g of pigment with a 1 cm optical path. In the actual detection process, the tested solution concentration often is not completely 1 %, and the optical path may not be exclusively 1 cm because of the solubility of the pigment and operational aspects. The measured pigment solution absorption value is different from the ratio of the absorption value. Therefore, the pigment solution absorption value needs to be transformed into the ratio of the absorption value. The method is as follows:

$$E = E_1 / C_1 L_1 \quad (4)$$

where E_1 was the measured absorption value of the pigment solution, C_1 was the measured concentration of the pigment solution, L_1 was the measured optical path of the pigment solution, E (CU) was the ratio of the absorption value of the pigment solution, C was the concentration of the pigment solution (1%), and L was the optical path of the pigment solution (1 cm).

The AEHP was diluted to 0.01 % with a citric acid disodium hydrogen phosphate buffer solution (pH 3).

$$E = A / (0.01 \times 1) \quad (5)$$

where A was the absorbance at λ_{max}.

2.5 Antioxidant Capacity of the Anthocyanin-Rich Extracts by the SP825 Resin

Anthocyanin possesses anti-inflammatory and anti-carcinogenic activity, cardiovascular disease prevention, obesity control, and diabetes alleviation properties, all of which are more or less associated with their potent antioxidant property (He et al., 2010). Antioxidant capacity of the anthocyanin-rich extracts was assayed according to the following methods.

2.5.1 DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the AEHP was measured according to the method described by Blois (2002) with some modifications (Blois, 2002). Aliquots (0.5 mL) of the various concentrations (0.039, 0.078, 0.156, 0.313, 0.625, 1.250 mg/mL) of the AEHP were mixed with 2 mL (25 μg/mL) of a MeOH solution of DPPH. Then, the mixture was shaken vigorously and allowed to stand in the dark for 30 min. The absorbance was measured at 517 nm against a blank. A decrease in the DPPH solution absorbance indicated an increase in the DPPH radical-scavenging activity, which was calculated according to the following equation:

$$DPPH\ radical\text{-}scavenging\ activity\ (\%) = [A_0 - A_1 / A_0] \times 100 \quad (6)$$

where A_0 was the absorbance without the sample and A_1 was the absorbance in the presence of the sample.

2.5.2 ABTS Radical Scavenging Assay

The ABTS was dissolved in distilled water at a final concentration of 7 mM and mixed with a potassium persulphate solution at a final concentration of 2.45 mM. The reaction mixture was left to settle at room temperature for 12-16 h in the dark before use (Trishna et al., 2011). For each experiment, a freshly prepared $ABTS^+$ solution was diluted with 0.01 M phosphate buffer saline (PBS, pH 7.4) to adjust its absorbance to within

0.70 ± 0.02 at 734 nm. Then, 0.15 mL of the samples with different concentrations (0.156-10.00 mg / mL) was mixed with 2.85 mL of the $ABTS^+$ solution. Finally, the absorbances were measured at 734 nm after incubation at room temperature for 10 min. The scavenging activity of the ABTS free radical was calculated using the following equation:

$$ABTS\ scavenging\ activity\ (\%) = [(C - D) - (A - B) / (C - D)] \times 100 \quad (7)$$

where A = absorbance of ABTS solution + sample / standard, B = absorbance of potassium persulphate + sample / standard, C = absorbance of ABTS solution + distilled water / methanol, and D = absorbance of potassium persulphate + distilled water / methanol.

2.5.3 Hydroxyl Radical Scavenging Activity

The HO• scavenging activity was measured according to a literature protocol with a few modifications (Nicholas, et al, 1989). The HO• was generated from $FeSO_4$ and H_2O_2 and detected by its ability to hydroxylate salicylate. The reaction mixture (2.5 mL) contained 0.5 mL of $FeSO_4$ (1.5 mM), 0.35 mL of H_2O_2 (6 mM), 0.15 mL of sodium salicylate (20 mM) and 1 mL of the AEHP solution at different concentrations. Ascorbic acid was used as the positive control. After incubation for 1 h at 37°C, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as:

$$\%\ HO\bullet\ scavenged = [1 - (A_1 - A_2) / A_0] \times 100\ \% \quad (8)$$

where A_1 was the absorbance of the sample or ascorbic acid, A_0 was the absorbance of the solvent control, and A_2 was the absorbance of the reagent blank without sodium salicylate.

2.5.4 Reducing Power

The reducing power of the AEHP was measured according to the method of Yen and Chen (1995) with slight modifications (Yen et al, 1995). An aliquot of each sample (1 mL), with different concentrations, was mixed with 1 mL of phosphate buffer (200 mM, pH 6.6) followed by 1 mL of 1% potassium ferricyanide ($K_3Fe[CN]_6$). The mixture was incubated for 20 min in a water bath at 50°C. After incubation, 1 mL of 1% trichloroacetic acid (TCA) was added. After straining, 2 mL of the sample solution from before was mixed with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride ($FeCl_3$), and then the absorbance was measured at 700 nm against a blank in the spectrophotometer. A higher absorbance indicates a higher reducing power activity. Ascorbic acid was used as the positive control.

2.5.5 Ferrous Metal Ions Chelating Ability

The ferrous metal ion chelating activity of the AEHP was measured according to a literature protocol with a few modifications (Decker et al, 1990). The sample or an ethylene diamine tetraacetic acid (EDTA) solution (1 mL) was mixed with 50 μL of ferrous chloride (2 mM) and 0.2 mL of ferrozine (5 mM). The mixtures were shaken well and allowed to stand for 10 min at room temperature, and the absorbance of the mixture was determined at 562 nm. EDTA was included as the positive control. The ion-chelating activity was calculated as:

$$Chelating\ rate\ (\%) = [1 - (A_1 - A_2) / A_0] \times 100\ \% \quad (9)$$

where A_0 was the absorbance of the control without the sample, A_1 was the absorbance in the presence of the sample, and A_2 was the absorbance without ferrozine.

2.5.6 SOD-like Activity

The levels of SOD-like activity in the AEHP were measured with the SOD Assay Kit-WST according to the technical manual provided by Dojindo Molecular Technologies, Inc. Briefly, in a 96-well plate, 20 μL of the sample solution was added to each sample and the well containing blank 2, and 20 μL of double distilled water was added to the wells containing blank 1 and blank 3. Then, 200 μL of the WST working solution was added to each well. After mixing, 20 μL of the dilution buffer was added to the wells containing blank 2 and blank 3, and 20 μL of the enzyme working solution was added to each sample and the well containing blank 1. The plate was incubated at 37°C for 20 min, and the O.D. was determined at 450 nm using a microplate reader (BIO-RAD Model 550, USA). The SOD-like activity was calculated by the following equation:

$$SOD\ activity\ (inhibition\ rate\ \%) = \{[(A_{blank\ 1} - A_{blank\ 3}) - (A_{sample} - A_{blank\ 2})] / (A_{blank\ 1} - A_{blank\ 3}) \times 100 \quad (10)$$

where $A_{blank\ 1}$, $A_{blank\ 2}$, $A_{blank\ 3}$, and A_{sample} were the absorbances of blank 1, blank 2, blank 3 and the samples, respectively.

2.6 Cell Culture

The human colorectal cancer cell line, DLD-1, the human bladder cancer cell line, EJ-1, and the murine

macrophage cell line, RAW 264.7, were purchased from the Riken Cell Bank (Tsukuba, Japan). The colorectal cancer cells were grown in RPMI 1640 (Roswell Park Memorial Institute-1640) medium. The other cells were grown in MEM medium (Minimum Essential Medium Eagle) containing 10% foetal bovine serum, 100 U / mL penicillin and 100 μg / mL streptomycin. The cells were cultured at 37°C in a humidified 5% CO_2 atmosphere (ESPEC CO_2 Incubator) for 2-3 days to reach the logarithmic phase for the following tests.

2.7 Cell Viability Assay

The inhibitory ability of the AEHP was measured on the human colorectal and bladder cancer cells. The cell cultures were exposed to various concentrations of the extracts for 24 and 48 h in Minimum Essential Medium (MEM) under anaerobic conditions at 37°C. The effects of the AEHP on the anti-proliferation of DLD-1 and EJ-1 cells and the effect of the AEHP on the proliferation of the RAW 264.7 cells were estimated with the Cell Counting Kit-8 (CCK-8). The cells were cultured in a 96-well plate at a density of 5×10^4 cells / mL at 37°C in a 5% CO_2 atmosphere for 24 h. Next, 10 μL of the AEHP solution, with concentrations of 0, 6.25, 12.5, 25, 50, 100, 200, 400 μg / mL, was added to the cell culture plates. The cell culture plates were cultured at 37°C in a 5% CO_2 atmosphere for 24 and 48 h. After this incubation, 10 μL of the CCK-8 solution was added, and the plates were incubated at 37°C for 4 h. The cell viability was determined by the O.D. at the wavelength of 450 nm with a microplate reader (BIO-RAD Model 550). The data were expressed as percentages of the control.

2.8 Statistical Analysis

The experiments were repeated three times, and each was arranged in three groups in parallel. The experimental results were given as the means ± S.D. of three replicate measurements. Tests of significant difference were determined by Student's t-test analysis at P = 0.05 or independent sample t-test (P = 0.05).

3. Results and Discussion

3.1 Static and Dynamic Adsorption / Desorption Tests

3.1.1 pH and Temperature

Table 1. The adsorption and degradation ratios of anthocyanins at different pHs (Static test data)

	A_{536nm}	pH								
		1	2	3	4	5	6	7	8	9
Adsorption ratio (%)	0.54	30.28	34.31	25.61	14.86	6.59	1.28	0.85	0.84	0.65
Degradation ratio (%)		66.59	51.32	45.29	34.70	23.36	12.69	11.75	10.71	6.65

Table 2. The yields of the AEHP and anthocyanin contents at different pHs (dynamic test data)

	pH		
	2	3	4
AEHP yield (mg)	84.00 ± 2.20	76.00 ± 1.90	54.00 ± 3.50
Anthocyanin content (mg)	24.51 ± 1.06	24.97 ± 1.19	12.93 ± 0.74
Anthocyanin concentration (%)	29.22 ± 2.05	32.83 ± 2.35	23.94 ± 2.81

As shown in Table 1, the maximum absorption wavelengths were all 536 nm from pH 1 to 9 except pH 2 and 3. At pH 2 and 3, the maximum absorption wavelengths were 534.5 and 535.5 nm, respectively. When the initial absorbance of the sample was 0.54 at 536 nm, the resin exhibited a stronger adsorption capacity and higher degradation ratio from pH 1 to 4 than from pH 5 to 9. According to the above results, the dynamic tests were investigated at pH 2, 3 and 4. Finally, the optimum pH was determined to be 3 (Table 2).

Next, the optimum temperature was evaluated by static tests at pH 3. The results showed that the adsorption capacity of the resin increased, and the degradation ratio of anthocyanins decreased as the temperature decreased (Zhao et al., 2011). Therefore, the optimum temperature was 25°C.

3.1.2 Adsorption Kinetics on SP825 Resin

The following kinetic models were adopted to describe the adsorption process: pseudo-first-order, pseudo-second-order and intra-particle diffusion kinetic models. The equations of the three models are the

following, respectively:

$$lg\ (q_e - q_t) = -\ (k_1 / 2.303)\ t + lg\ q_e \quad (11)$$

$$t / q_t = (1 / q_e)\ t + 1 / k_2\ q_e^2 \quad (12)$$

$$q_t = k_{id}\ t^{1/2} + c \quad (13)$$

where q_e and q_t were the adsorption capacity at equilibrium and at any time t (mg / g dry resin), respectively. The parameters k_1 (min^{-1}), k_2 (g/[mg·min]) and k_{id} (mg / [g·$min^{1/2}$]) were the apparent rate constants of the pseudo-first-order, pseudo-second-order and intra-particle diffusion models for the adsorption process, respectively. C (mg / g), which was the constant, represents the boundary layer thickness. Plotting lg (q_e - q_t) against t produced straight lines (R^2 = 0.4653) for Equation (11). Plotting t / q_t against t produced straight lines (R^2 = 0.9919, y = 0.1148 x + 10.526) for Equation (12). Plotting q_t versus $t^{1/2}$ produced straight lines (R^2 = 0.9704) for equation (13). The pseudo-second-order kinetic model was suitable for describing the whole adsorption process of the anthocyanins on the SP825 resin. Furthermore, the finding implied that concentrations of both the adsorbate and adsorbent were involved in the rate-determining step in the adsorption process, which might be chemical adsorption or chemisorption (Liu et al., 2010).

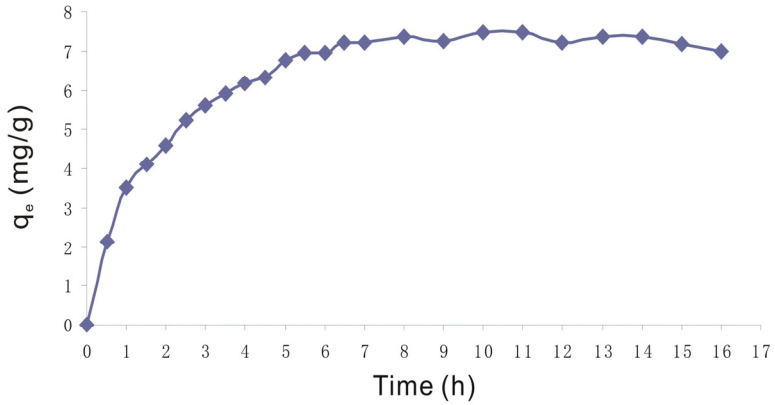

Figure 1. Adsorption kinetic curves for anthocyanins on the SP825 resin at 25°C

As shown in Figure 1, at 25°C, the absorption kinetic curve of the anthocyanins on the SP825 resin was obtained. The absorption capacity increased with increased time. The absorption reached equilibrium after 8 h (q_e = 8.71 mg / g).

3.1.3 Adsorption Isotherms and Thermodynamics on the SP825 Resin

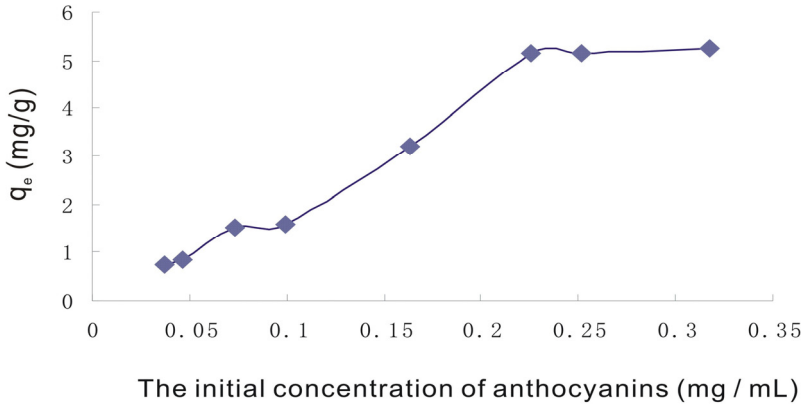

Figure 2. Adsorption isotherms for anthocyanins on the SP825 resin at 25 °C

As shown in Figure 2, the equilibrium adsorption isotherm was constructed at 25 °C. The initial concentrations of the anthocyanins were 0.037, 0.047, 0.073, 0.099, 0.163, 0.225, 0.252, and 0.318 mg / mL, respectively. The

absorption capacity increased as the concentration increased. Moreover, the resin reached the saturation plateau after 8 h in three samples, which contained 0.225, 0.252, and 0.318 mg / mL anthocyanins. Thus, in theory, they were selected as the initial concentrations to extract.

The equilibrium data were analysed following the Langmuir and Freundlich model. The Langmuir equation $C_e / q_e = C_e / q_m + (1 / K) q_m$ was converted to the linearised form with C_e and C_e / q_e as independent variables and $R^2 = 0.7541$. A linearised version of the Freundlich equation $q_e = K C_e^{1/n}$ can be written as equation $\log q_e = \log K + (1/n) \log C_e$, with K and n obtained from the intercept and slope (K = 23.8, n = 1), respectively, and $R^2 = 0.9672$. In these equations, q_e was the adsorption capacity at equilibrium (mg / g dry resin), C_e was the concentration of anthocyanins at equilibrium (mg / mL), and q_m was the maximum adsorption capacity (mg / g dry resin).

The Langmuir and Freundlich equations are used to reveal the linearity fit and to describe the equilibrated relationship between the concentrations of the adsorbate in the fluid phase and the adsorbent at a given temperature (Liu et al., 2010). The absorption process is consistent with the Freundlich model (n = 1). Generally, adsorption is difficult when n is less than 0.5.

3.1.4 Dilution Multiple of Sample Solution

The theoretical concentrations of the samples were obtained from isothermal adsorption experiments at a natural pH. In the dynamic tests, the volumes of added distilled water were 1, 2, 3 and 4 sample volumes, and the dilution multiples were 2, 3, 4 and 5, respectively. The anthocyanin contents of the AEHP were 2.5 ± 0.34 mg, 3.3 ± 0.48 mg, 2.2 ± 0.09 mg and 0.9 ± 0.23 mg, respectively. The optimum dilution multiple of the sample was 3.

3.1.5 The Concentration of Ethanol Eluting Agent

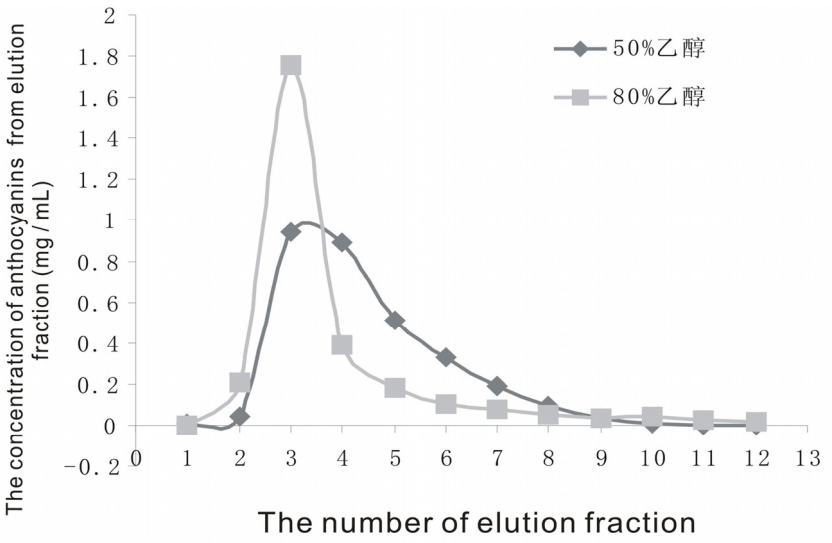

Figure 3. Ethanol elution curves for anthocyanins in the SP825 resin at 25°C

As shown in Figure 3, the volume of every elution fraction was 2.5 mL. The horizontal coordinate expressed the numbers of the elution fractions over time. The ethanol eluting agent concentrations were 50% and 80%. The elution fraction volume was 2.5 mL. The elution curve was drawn according to the anthocyanin concentration of the fraction. Figure 3 showed that the anthocyanins were more concentrated with the 80% ethanol elution agent, meaning that the sample volume became smaller and the rotary evaporating time became shorter. Finally, the loss of the anthocyanins decreased in the extraction process. Thus 80% ethanol was found to be able to elute most of the anthocyanins adsorbed by the resin (Liu et al., 2004).

3.2 Anthocyanins and Polyphenol Contents and E of AEHP

A total of 1000 g of *H. polyrhizus* fresh fruit (both peel and pulp) contained 658 mg of anthocyanins and could be made into 1.5 g of the AEHP, which contained 520 mg of anthocyanins. Thus, 79% of the anthocyanins could be extracted from the fresh fruit.

In the following experiments, we used an AEHP containing 29% anthocyanins and 31.3% polyphenol, and its E was 76.23. According to the International colour units (ICU) standard, E = 150 is equivalent to 100000 ICU. The

E of the AEHP was 76.23, equivalent to 50820 ICU. The E of the AEHP was 19 times that of the food additive Radish red GB6718-86. Thus, the AEHP has a strong potential as a natural colorant.

3.3 Antioxidant Abilities of AEHP

Table 3. EC_{50} value and anthocyanin and polyphenol contents at the EC_{50} value

Antioxidant assays	EC_{50} of positive control (mg / mL)	EC_{50} of AEHP (mg / mL)	Anthocyanin content of AEHP (mg / mL)	Polyphenol content of AEHP (mg / mL)	Positive control
DPPH·radical scavenging activity	0.290	0.040	0.012	0.013	Ascorbic acid
ABTS radical scavenging activity	0.840	0.170	0.049	0.053	Ascorbic acid
Ferrous metal ions chelating activity	1.260	0.540	0.157	1.725	EDTA
SOD	——	0.170	0.049	0.053	——

As shown in Table 3, the antioxidant abilities of the AEHP were analysed. The values of EC_{50} were obtained by interpolation from linear regression analysis. The values of EC_{50} were the effective concentrations when 50% radicals were scavenged.

3.3.1 DPPH·Radical Scavenging Activity

The DPPH·approach is simple and widely applied to the measurement of the antioxidant activity of polyphenolics and colourants (Lichen Wu et al., 2006). With ascorbic acid as a positive control, the EC_{50} values of ascorbic acid and the AEHP were 290 μg / mL (R^2 = 1) and 40 μg / mL (R^2 = 0.96), respectively. The consumption of AEHP was 14.3 % of ascorbic acid scavenging 50 % of the radicals. Compared with the results of the anthocyanin-rich extract from acai at 50 μg / mL quenching 39.6 % of the radicals (Hogan et al., 2010), AEHP had a better efficiency in the DPPH· radical scavenging activity. This finding suggested that the AEHP might have biomedical applications in reducing body oxidative stress and the deteriorating situation arising from the elevation of various reactive oxygen species (ROS).

3.3.2 ABTS Radical Scavenging Activity

Anthocyanins are water-soluble pigments (Benoit, 2004). The ABTS method is suitable for not only water-soluble antioxidants but also lipid-soluble antioxidants. The ABTS approach has been suggested to be better for evaluating the antioxidant activity of phenolic phytochemicals than the DPPH radical scavenging assay (Wu et al., 2006). With ascorbic acid as a positive control, the EC_{50} values of ascorbic acid and AEHP were 840 μg / mL (R^2 = 0.9997) and 170 μg / mL (R^2 = 0.9527), respectively. The consumption of AEHP was 20% of ascorbic acid scavenging 50 % of the radicals.

3.3.3 Hydroxyl Radical Scavenging Activity

The hydroxyl radical is considered the most reactive and poisonous free radical in organisms because it can non-specifically oxidise all classes of biological macromolecules, including lipids, proteins, and nucleic acids (Xiong et al., 2011). When the dose was 0.156 mg / mL, the hydroxyl scavenging ratios of the positive control (ascorbic acid) and AEHP were 6.67 % and 100 %, respectively. The hydroxyl radical scavenging ability of the AEHP was 15 times that of ascorbic acid.

3.3.4 Reducing Power

Table 4. Absorbance at 700 nm

Dose (mg / mL)	0.156	0.313	0.625	1.25	2.5	5
Ascorbic acid	0.853 ± 0.015	1.074 ± 0.039	1.490 ± 0.011	1.579 ± 0.010	1.611 ± 0.007	1.668 ± 0.001
AEHP	0.111 ± 0.000	0.169 ± 0.010	0.619 ± 0.020	1.242 ± 0.010	1.643 ± 0.000	1.661 ± 0.000

The reducing power shows the electron-donating activity. A strong reducing power indicates a good electronic supply capacity. It may be used as an indicator of potential antioxidant activity (Wang et al., 2008). Table 4 presents the reducing power of the AEHP and the positive control (ascorbic acid). A great O.D. at 700 nm showed a great reduction force and suggested that the AEHP has a dose-dependent reducing power. At doses of less than 2.5 mg / mL, the reducing power of the AEHP increased at a faster speed than that of ascorbic acid. At doses of greater than 2.5 mg / mL, the reducing powers of the AEHP and ascorbic acid were approximately the same.

3.3.5 Ferrous Metal Ion Chelating Activity

The transition metal can be used as a catalyst to produce the initiated few free radicals that mediate the oxidation chain reaction in biological and food systems. The concentration of the transition metal can be reduced because of the metal chelating ability. In this study, the AEHP was compared with a positive control (EDTA). The chelating action of the AEHP on the ferrous ions increased with the increasing concentration of the AEHP. The EC_{50} values of EDTA and the AEHP were 1260 μg / mL ($R^2 = 0.99$) and 540 μg / mL ($R^2 = 0.96$), respectively. The consumption of the AEHP was less than 50% of EDTA chelating 50% of the ferrous metal ions. EDTA chelated 59.16 ± 4.62% of the ferrous ions at 1.25 mg / mL, and the AEHP showed a stronger chelating ability (89.50 ± 1.10 %).

3.3.6 SOD-Like Activity of AEHP

All organisms have a complex antioxidant defence system, including antioxidant enzymes, such as SOD and catalase (Shi et al., 2013). The AEHP demonstrated a strong SOD-like activity with its increased concentration, and its SOD-like activity was 100% at 1.25 mg / mL. The anthocyanin and polyphenol contents were 0.363 and 0.391 mg / mL, respectively.

3.4 Anticancer Ability of AEHP

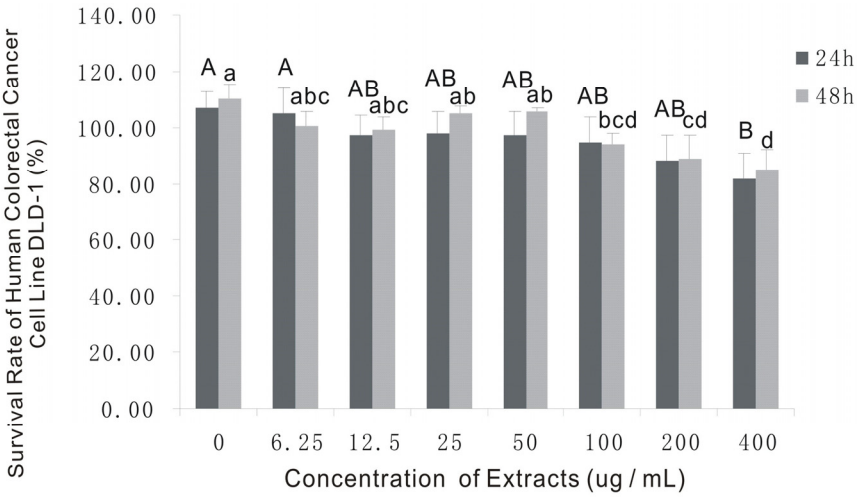

Figure 4. The effect of the AEHP on the survival ratios of human colorectal cancer cells, DLD-1

In Figure 4, the capital letter group and the small letter group represent significant differences in the cancer cell survival ratios, depending on the AEHP treatment at different concentrations, after 24 and 48 h, respectively. The same letters represented no significant difference in the groups, and different letters represented the opposite. As shown in Figure 4, the AEHP could inhibit the proliferation of the human colorectal cancer cell line, DLD-1. The DLD-1 cell survival ratio showed significant differences under the AEHP concentrations between 0 and 400 μg / mL after 24 h ($p < 0.05$). Moreover, the DLD-1 cell survival ratios presented significant differences between the concentrations of 100, 200 and 400 μg / mL and 0 μg / mL AEHP after 48 h ($p < 0.05$).

Table 5. Time and concentration double factors analysis results of AEHP on human colorectal cancer cells

Impact factors	Df	F	Siq
Concentration	7	8.312	0.000
Time	1	1.506	0.229
Concentration×Time	7	0.512	0.819

As Table 5 shows, the concentration was the key factor to inhibit the growth of the cancer cells, but time was not.

Otherwise, the AEHP could not inhibit the proliferation of the human bladder cancer cell line EJ-1. These results indicated that biological extract might have a different inhibitory mechanism against the proliferation of cancer cell, suggesting that the specificity of the bioactive components should be studied.

3.5 Macrophage Proliferation by AEHP

The AEHP shows a proliferation effect on the macrophage RAW 264.7 cells. After treatments with 200 and 400 μg / mL AEHP for 24 h, the macrophage survival ratios were 168.82% and 178.12%, respectively. However, the macrophage survival ratios were decreased by 32.26% and 26.65%, respectively, after 48 h. AEHP affected macrophages growth in a dose-dependent and time-dependent manner.

4. Conclusions

Using a dilution multiple of 3 and pH of 3, Purple pitaya (*H. polyrhizus*) juice were filtered through 300 mesh gauze. Then diatomite powders were placed into the centrifuge. Next, the supernatant, 3 BV distilled water and 3 BV ethanol eluting agent (80%) were passed through the resin column at 3 mL / min in that order. The collected elution liquid was rotated and evaporated and then freeze-dried. We extracted 79% of the anthocyanins from the fresh fruits. In addition, the AEHP revealed strong DPPH radical scavenging activity, ABTS radical scavenging activity, hydroxyl radical scavenging, reducing power, ferrous metal ion chelating activity and SOD-like activity. Moreover, the AEHP inhibited the proliferation of cancer cells and promoted the proliferation of the macrophage cells. Further studies are in progress on the characterisation and functional effects of the anthocyanins from the AEHP and their activation mechanism.

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