Extraction of Anthocyanins from Carmine Radish and Their Effect on NCI-N87 Cell Proliferation and Invasion (Postprint)

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Abstract

To investigate the extraction process of anthocyanins from red radish, the effects of liquid-to-material ratio, extraction temperature, extraction time, ultrasonic power, and ultrasonic time on the anthocyanin extraction yield were examined. The effects of anthocyanins on the proliferation and invasion of human gastric cancer cell line NCI-N87 were studied using MTT assay and cell invasion assay, and their influence on the HER2 signaling pathway was further analyzed by Western blotting. The results showed that, based on response surface analysis, the optimal extraction conditions for red radish anthocyanins were: using 1% hydrochloric acid ethanol as the extraction solvent, a liquid-to-material ratio of 10:1, extraction at 40°C for 2 h, and ultrasonic disruption at 400 W for 15 min. Under these conditions, the maximum anthocyanin extraction amount reached 3.92 mg/g. Red radish anthocyanins at 30 g/mL could significantly inhibit the proliferation and invasion capabilities of NCI-N87 cells, while simultaneously suppressing the phosphorylation levels of HER2 protein and Akt.

Full Text

Extraction of Anthocyanins from Carmine Radish and Their Effects on NCI-N87 Cell Proliferation and Invasion

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Abstract

This study investigated the extraction process of anthocyanins from carmine radish by examining the effects of solid-liquid ratio, extraction temperature, ex-

traction time, ultrasonic power, and ultrasonic duration on extraction yield. The effects of anthocyanins on the proliferation and invasion of human gastric cancer NCI-N87 cells were evaluated using MTT assays and cell invasion experiments, with Western blot analysis employed to assess impacts on the HER2 signaling pathway. Response surface methodology revealed the optimal extraction conditions: 1% hydrochloric acid-ethanol as the extraction solvent, a solid-liquid ratio of 10:1, extraction at 40°C for 2 hours, followed by ultrasonic disruption at 400W for 15 minutes. Under these conditions, the maximum anthocyanin yield reached 3.92 mg/g. Treatment with 30 g/mL of carmine radish anthocyanins significantly inhibited NCI-N87 cell proliferation and invasion while suppressing HER2 protein expression and Akt phosphorylation levels.

Keywords: carmine radish; anthocyanin; cell proliferation; cell invasion

Introduction

Anthocyanins are flavonoid compounds present in plant vacuoles, with natural anthocyanins classified into cyanidin, pelargonidin, delphinidin, peonidin, malvidin, and petunidin [1]. Research has demonstrated that anthocyanins possess anti-tumor activity, primarily through modulation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase-serine/threonine kinase (PI3K-Akt) pathways, thereby inhibiting tumor cell proliferation and invasion while promoting apoptosis [2-4]. Gastric cancer is a malignant tumor derived from epithelial tissue, with approximately 20% of cases characterized by overexpression of human epidermal growth factor receptor 2 (HER2). Carmine radish (*Carmine radish*), a characteristic vegetable of Fuling, Chongqing, is rich in anthocyanins, with pelargonidin accounting for 95% of the total content [5-8]. This study established an optimized ultrasonic-assisted extraction protocol for carmine radish anthocyanins and investigated their effects on the proliferation and invasion of the gastric cancer cell line NCI-N87, along with their influence on HER2 signaling pathway-related proteins.

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Materials and Methods

1.1 Reagents and Instruments

Carmine radish (Carmine Red No. 1) was used as the raw material. NCI-N87 cells were obtained from the American Type Culture Collection (ATCC). Trypsin (180 units), bovine serum albumin (BSA), and ECL developing solution were purchased from Millipore. Protein marker and phosphate-buffered saline (PBS) were from TaKaRa. PVDF membrane, Tris-HCl (pH 6.8, pH 8.8), sodium dodecyl sulfate (SDS) solution, ACR (acrylate copolymer) solution, and crystal violet were from Bio-Rad. RPMI-1640 medium was from Hyclone. MTT assay kit (C0009) and HER2 antibody (AF0177) were from Beyotime. Rabbit

anti-AKT (380617), rabbit anti-p-AKT (Ser473) (381555), rabbit anti-GAPDH (380626), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (511201) were from Zhengneng Biological. All other chemical reagents were of analytical grade.

The GelDoc XR+ gel imaging system was from Bio-Rad (USA). The TS100 inverted phase-contrast microscope was from Nikon (Japan). The MK3 microplate reader was from Thermo (USA). The JY98-II ultrasonic cell crusher was from Ningbo Xinzhi. The T6 UV-Vis spectrophotometer was from Beijing Puxi.

1.2.1 Ultrasonic-Assisted Extraction of Anthocyanins

Carmine radish slices were dried at 60°C to constant weight, pulverized, and passed through a 40-mesh sieve before being stored away from light at room temperature. One gram of radish powder was weighed and extracted with 1% hydrochloric acid-ethanol as the solvent. The solid-liquid ratio was adjusted, and the mixture was extracted at a specified temperature, followed by ultrasonic treatment at varying power levels and durations. The solvent was removed using a rotary evaporator, and anthocyanins were dissolved in deionized water for content determination [9] using the following formula:

Where p represents anthocyanin content (mg/g); A and A are the absorbance values at 520 nm at pH 1.0 and pH 4.5, respectively; V is the total extraction volume (mL); n is the dilution factor; M is the relative molecular mass of pelargonidin-3-sophoroside (648.5); is the molar extinction coefficient (30200); and m is the sample mass (g).

1.2.2 Single-Factor Experimental Design

The effects of various parameters on anthocyanin extraction yield were investigated: solid-liquid ratios of 5:1, 10:1, 15:1, 20:1, and 25:1; extraction temperatures of 20°C, 30°C, 40°C, 50°C, and 60°C; extraction times of 1 h, 1.5 h, 2 h, 2.5 h, and 3 h; ultrasonic power levels of 100W, 200W, 300W, 400W, and 500W; and ultrasonic durations of 5 min, 15 min, 25 min, 35 min, and 45 min.

1.2.3 Response Surface Methodology Experimental Design

Based on single-factor results, extraction time (A), ultrasonic power (B), and extraction temperature (C) were selected as independent variables, with anthocyanin extraction yield as the response value to optimize extraction conditions. The factor levels are shown in Table 1 .

Table 1 Factors and levels for response surface analysis

Factor	Level 1	Level 2	Level 3
Extraction time (A) / h	-	-	-
Ultrasonic power (B) / W	-	-	-

Factor	Level 1	Level 2	Level 3
Extraction temperature (C) / °C	-	-	-

1.2.4 Purification of Anthocyanins by Macroporous Resin

Pre-treated AB-8 macroporous resin was packed into a chromatography column. Anthocyanin extract was loaded onto the column and eluted with 1% hydrochloric acid-ethanol. The eluate was collected, and ethanol was recovered at 40°C using a rotary evaporator to obtain purified anthocyanins.

1.2.5 Cell Culture

NCI-N87 cells were purchased from ATCC and maintained in our laboratory. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO incubator. Upon reaching 80-90% confluence, cells were digested with 0.25% trypsin and passaged.

1.2.6 MTT Assay

The assay was performed according to the manufacturer' s instructions for the Beyotime Cell Proliferation and Cytotoxicity Detection Kit (C0009). Briefly, 100 µL of NCI-N87 cell suspension (2000 cells) was added to each well of a 96-well plate and incubated for 24 h at 37°C in a 5% CO incubator. Cells were treated with various concentrations of anthocyanins (10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL) or 0.1% DMSO as control for 24 h, 48 h, or 72 h. Subsequently, 10 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h, followed by addition of 100 µL of solubilization solution and another 4 h incubation. Absorbance was measured at 570 nm using a microplate reader. The experiment was repeated three times with three parallel samples each time.

1.2.7 Cell Invasion Assay

NCI-N87 cells were digested with 0.25% trypsin, and 5×10 cells in 200 µL medium were seeded into invasion chambers. Cells were treated with various anthocyanin concentrations (10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL) or 0.1% DMSO as control. The chambers were placed in 24-well plates containing 700 µL of culture medium. After 12 h incubation, chambers were removed, culture medium was aspirated, and cells were fixed with formaldehyde for 15 min. Following fixation, cells were stained with 0.1% crystal violet for 10 min, washed three times with PBS, and cells on the outer bottom surface were removed. Three random fields were photographed at 20× magnification under an inverted microscope and counted. The experiment was repeated three times.

1.2.8 Western Blot Analysis of Protein Expression

NCI-N87 cells were seeded in 6-well plates. Upon reaching 90% confluence, cells were treated with various anthocyanin concentrations (10 μg/mL, 20 μg/mL, 30 μg/mL, 40 μg/mL) or 0.1% DMSO as control for 24 h. Cells were then digested and collected, total protein was extracted, and 50 μg of protein was loaded per well. Proteins were separated by SDS-PAGE at 200V, transferred to membranes, and blocked for 1 h. Membranes were incubated overnight at 4°C with primary antibodies against HER2, p-Akt, Akt, and GAPDH, followed by incubation with secondary antibody for 1 h at room temperature. After ECL development, appropriate exposure times were selected for imaging and analysis.

1.2.9 Statistical Analysis

All experimental data are presented as mean ± standard deviation ($\bar{x} \pm s$). Statistical analysis was performed using SPSS 22.0 software. Comparisons between two groups were conducted using analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

2.1.1 Effect of Solid-Liquid Ratio on Anthocyanin Extraction Yield

As shown in Figure 1 [Figure 1: see original paper], at solid-liquid ratios below 10:1, increased ethanol volume enhanced contact with radish powder and improved anthocyanin solubility [10-11]. However, when the ratio exceeded 10:1, anthocyanin extraction yield decreased with further increases in solvent volume, indicating that solubility had reached its maximum. Additional solvent would only increase production costs without improving yield. Therefore, a solid-liquid ratio of 10:1 was selected as optimal.

Figure 1 Effect of solid-liquid ratio on anthocyanin content

2.1.2 Effect of Extraction Time on Anthocyanin Extraction Yield

Figure 2 [Figure 2: see original paper] demonstrates that anthocyanin extraction yield increased with extraction time up to 2 h, after which it began to decline. This may be attributed to anthocyanin degradation due to prolonged exposure to environmental factors [11]. Thus, an extraction time of approximately 2 h was deemed appropriate.

Figure 2 Effect of extraction time on anthocyanin content

2.1.3 Effect of Extraction Temperature on Anthocyanin Extraction Yield

As illustrated in Figure 3 [Figure 3: see original paper], anthocyanin extraction yield increased with temperature up to 40°C, but decreased significantly

at higher temperatures. Anthocyanin stability is highly temperature-sensitive, and elevated temperatures can disrupt their monomeric structure [12] while potentially co-extracting other radish components that reduce purity. Therefore, 40°C was selected as the optimal extraction temperature.

Figure 3 Effect of extraction temperature on anthocyanin content

2.1.4 Effect of Ultrasonic Duration on Anthocyanin Extraction Yield

Figure 4 [Figure 4: see original paper] shows that ultrasonic duration had a relatively minor effect on extraction yield, with maximum yield achieved between 15-25 min. Further extension of ultrasonic time did not significantly improve yield, so 15 min was selected as the optimal duration.

Figure 4 Effect of ultrasonic time on anthocyanin content

2.1.5 Effect of Ultrasonic Power on Anthocyanin Extraction Yield

As depicted in Figure 5 [Figure 5: see original paper], anthocyanin extraction yield increased with ultrasonic power, reaching maximum at 400W. At higher power levels, increased solubility of non-anthocyanin components may have reduced the apparent anthocyanin content. Therefore, 400W was determined to be the optimal ultrasonic power.

Figure 5 Effect of ultrasonic power on anthocyanin content

2.2.1 Establishment and Significance Testing of the Regression Model

Based on single-factor experiments, extraction time (A), ultrasonic power (B), and extraction temperature (C) were selected for response surface analysis to identify the optimal combination. The experimental results are presented in Table 2 (three replicates). Using Design Expert 8.0 software, multiple regression analysis was performed with anthocyanin extraction yield (mg/g) as the response value (Y), yielding the regression equation:

$$Y = 3.92 + 0.27\times A - 0.013\times B + 0.14\times C - 0.42\times A^2 - 0.098\times B^2 - 0.28\times C^2$$

Table 2 Response surface analysis design and experimental results

Variance analysis results (Table 3) indicated that the regression model was highly significant ($P < 0.0001$) with a non-significant lack-of-fit term (0.4164), demonstrating good model fit and practical applicability. Ultrasonic power (B) showed relatively minor influence on extraction yield ($P > 0.0001$). After removing non-significant terms, the optimized equation became:

$$Y = 3.92 + 0.27\times A + 0.14\times C - 0.42\times A^2 - 0.098\times B^2 - 0.28\times C^2$$

Table 3 Variance analysis of regression model

Source	Sum of Squares	df	Mean Square	F-value	P-value
Model	-	-	-	-	< 0.0001
A	-	-	-	-	< 0.0001
B	-	-	-	-	< 0.0001
C	-	-	-	-	< 0.0001
A^2	-	-	-	-	< 0.0001
B^2	-	-	-	-	< 0.0001
C^2	-	-	-	-	< 0.0001
$R^2 = 0.9945$					

Note: * indicates $P < 0.0001$

Analysis of Figure 6 [Figure 6: see original paper] revealed that extraction time and temperature had substantial effects on anthocyanin yield. Response surface optimization identified the theoretical optimal conditions as: extraction temperature 42.22°C, extraction time 2.11 h, and ultrasonic power 386.67W, with a predicted maximum yield of 3.976 mg/g. To validate these conditions and facilitate practical application, parameters were adjusted to: extraction temperature 40°C, ultrasonic power 400W, and extraction time 2 h. Five replicate experiments under these conditions yielded 3.92 ± 0.0224 mg/g, confirming the stability and reliability of the optimized extraction protocol.

Figure 6 Response surface analysis results (a: extraction time and temperature; b: extraction time and ultrasonic power; c: extraction time and ultrasonic power)

2.3.1 Anthocyanin Inhibition of NCI-N87 Cell Proliferation

Previous studies have reported that crude anthocyanin extracts and purified compounds from fruits and vegetables can inhibit proliferation of various tumor cell types [13-14]. MTT assay results (Table 4) showed that 10 μg/mL anthocyanins had no significant effect on cell proliferation, while concentrations of 20 μg/mL, 30 μg/mL, and 40 μg/mL inhibited proliferation. Both 30 μg/mL and 40 μg/mL treatments showed significant differences compared to the control group ($P < 0.05$), with no significant difference between these two concentrations, indicating that 30 μg/mL carmine radish anthocyanins exerted a substantial inhibitory effect on cell proliferation.

Table 4 Effects of anthocyanins on NCI-N87 cell proliferation (mean ± s) (absorbance)

Treatment	24 h	48 h	72 h
Control	1.02 ± 0.11	1.05 ± 0.12	1.08 ± 0.11
10 μg/mL	0.92 ± 0.08	0.94 ± 0.10	0.96 ± 0.11
20 μg/mL	0.78 ± 0.09*	0.80 ± 0.10*	0.82 ± 0.08*#

Treatment	24 h	48 h	72 h
30 μg/mL	0.65 ± 0.07*	0.66 ± 0.06*#	0.68 ± 0.05*#Δ
40 μg/mL	0.63 ± 0.06*	0.61 ± 0.08*	0.70 ± 0.07*#

Note: * indicates $P < 0.05$ vs. control; # indicates $P < 0.05$ vs. 24 h same dose; Δ indicates $P < 0.05$ vs. 48 h same dose.

2.3.2 Anthocyanin Inhibition of NCI-N87 Cell Invasion

Cell invasion assay results (Figure 7 [Figure 7: see original paper] and Figure 8 [Figure 8: see original paper]) demonstrated that invasion capacity gradually decreased with increasing anthocyanin concentration. While 10 μg/mL and 20 μg/mL treatments showed no significant difference from the control, both 30 μg/mL and 40 μg/mL treatments exhibited significant inhibition ($P < 0.05$) with no significant difference between these two concentrations, indicating that 30 μg/mL anthocyanins produced a substantial inhibitory effect on cell invasion.

Figure 7 Effects of anthocyanins on NCI-N87 cell invasion capacity

Figure 8 Statistical analysis of anthocyanin effects on NCI-N87 cell invasion

Note: * indicates $P < 0.05$ vs. control

2.3.3 Anthocyanin Inhibition of the HER2 Signaling Pathway

HER2 is a transmembrane protein with tyrosine protein kinase activity that belongs to the epidermal growth factor receptor (EGFR) family. It can induce tumorigenesis and metastasis by phosphorylating downstream Akt protein [15-16]. Anthocyanins have been shown to inhibit migration and invasion of HER2-overexpressing breast cancer cell lines (BT474, MDA-MB-231, MCF-7) by suppressing HER2 and downstream signaling [17-18]. In this study, Western blot analysis revealed (Figure 9 [Figure 9: see original paper]) that 10 μg/mL anthocyanin treatment did not significantly affect HER2 or p-Akt protein expression, while 20 μg/mL, 30 μg/mL, and 40 μg/mL treatments inhibited both HER2 and p-Akt expression, with no significant changes in total Akt protein levels. These results suggest that carmine radish anthocyanins inhibit invasion of HER2-overexpressing gastric cancer NCI-N87 cells, likely through modulation of HER2 protein expression and Akt phosphorylation.

Figure 9 Effects of anthocyanins on HER2 and Akt protein expression

Discussion

This study employed Box-Behnken central composite design and response surface analysis to establish a quadratic polynomial mathematical model for ultrasonic extraction of carmine radish anthocyanins. The model was validated as reasonable and reliable for optimizing anthocyanin extraction yield. The

optimal ultrasonic-assisted extraction parameters were: 1% hydrochloric acid-ethanol as extraction solvent, solid-liquid ratio 10:1, extraction temperature 40°C, ultrasonic power 400W, and extraction time 2 h, achieving a maximum anthocyanin yield of 3.92 mg/g. Further investigation of the anti-tumor activity of anthocyanins purified by AB-8 macroporous resin revealed that 30 μg/mL carmine radish anthocyanins inhibited proliferation and invasion of gastric cancer NCI-N87 cells while reducing HER2 and phosphorylated Akt protein expression. These findings indicate that carmine radish anthocyanins can suppress proliferation and invasion of HER2-positive gastric cancer cells through the HER2-Akt signaling pathway. Future studies utilizing tumor xenograft mouse models and isolating pelargonidin, the main component of carmine radish, will help determine whether it exhibits more potent anti-proliferative and anti-invasive effects, providing theoretical support for developing anthocyanin products from this variety.

References

[1] Jeong J W, Lee W S, Shin S C, et al. Anthocyanins downregulate lipopolysaccharide induced inflammatory responses in BV2 microglial cells by suppressing the NF- B and Akt/MAPKs signaling pathways[J]. International Journal of Molecular Sciences, 2013, 14(1): 1502-1515.

[2] Shin D Y, Lee W S, Lu Jingnan, et al. Induction of apoptosis in human colon cancer HCT-116 cells by anthocyanins through suppression of Akt and activation of p38-MAPK[J]. International Journal of Oncology, 2009, 35(6): 1499-1504.

[3] Oak M H, Bedoui J E, Madeira S V F, et al. Delphinidin and cyanidin inhibit PDGFAB-induced VEGF release in vascular smooth muscle cells by preventing activation of p38 MAPK and JNK[J]. British Journal of Pharmacology, 2006, 149(3): 283-290.

[4] Huang Huipei, Shih Y W, Chang Y C, et al. Chemoinhibitory effect of mulberry anthocyanins on melanoma metastasis involved in the Ras/PI3K pathway[J]. Journal of Agricultural and Food Chemistry, 2008, 56(19): 9286-9293.

[5] Shi Jianwei, Teng Xiaoxu, Cao Tuanwu. Research progress on extraction, separation and purification of red pigment from Fuling carmine radish[J]. Guangzhou Chemical Industry, 2016, 2: 6-7.

[6] Rukiya, Sawut, Zhao Lifeng. Extraction and analysis of red pigment from Fuling carmine radish[J]. Journal of Kashgar Teachers College, 2005, 26(3): 61-63.

[7] Liang Shan, Jiang Zichuan, Yang Xia, et al. Determination of pelargonidin in carmine radish by high-performance liquid chromatography[J]. Guangzhou Chemical Industry, 2017, 45(7): 92-93.

[8] Badumala. Study on ultrasonic-assisted extraction of anthocyanins from red-heart radish[J]. Food Safety Guide, 2015, 33: 135-138.

[9] Song Dequn, Meng Xianjun, Wang Chenyang, et al. Determination of blueberry anthocyanins by pH-differential method[J]. Journal of Shenyang Agricultural University, 2013, 44(2): 231-233.

[10] Rui Chunmei, Li Chengqiong, Si Jun. Principles and methods of carmine radish red pigment extraction[J]. Southern Agriculture, 2007, 1(1): 71-72.

[11] Yang Hui, Zeng Zhaoyin. Study on ultrasonic-assisted extraction of carmine radish pigment[J]. Science and Technology of Food Industry, 2012, 33(24): 325-327.

[12] Chen Jianbo, Kuang Shoumin, Meng Juguang. Study on microwave-assisted extraction of radish red pigment[J]. Cereals and Oils Processing, 2008, 3: 124-126.

[13] Li Y W, Wang D, Li X G, et al. Anthocyanins extracted from Chinese blueberry and its anticancer effects on HepG2 cells[J]. Advanced Materials Research, 2014, 2989(887): 592-595.

[14] A Johnson S, H Arjmandi B. Evidence for anti-cancer properties of blueberries: a mini-review[J]. Anti-Cancer Agents in Medicinal Chemistry, 2013, 13(8): 1142-1144.

[15] Lordick F, Al-Batran S E, Dietel M, et al. HER2 testing in gastric cancer: results of a German expert meeting[J]. Journal of Cancer Research & Clinical Oncology, 2017, 143(5): 835-841.

[16] Güttlein L N, Benedetti L G, Fresno C, et al. Predictive outcomes for HER2-enriched cancer using growth and metastasis signatures driven by SPARC[J]. Molecular Cancer Research, 2017, 15(3): 304.

[17] Ozbay T, Nahta R. Delphinidin inhibits HER2 and Erk1/2 signaling and suppresses growth of HER2-overexpressing and triple negative breast cancer cell lines[J]. Breast Cancer: Basic and Clinical Research, 2011, 5: 143-154.

[18] Xu Mei, Bower K A, Wang Siying, et al. Cyanidin-3-glucoside inhibits ethanol-induced invasion of breast cancer cells overexpressing ErbB2[J]. Molecular Cancer, 2010, 9(1): 285.

Note: Figure translations are in progress. See original paper for figures.