Cytotoxic, Antioxidant and Antifatigue Activities of 8 Cultivated Varieties *myricarubra* Kernel and Residue Extracts

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Abstract The development of bayberry product has been long restricted to the under utilised residue and the waste of the kernels. In this paper, we aimed to investigate whether the waste part of bayberry produced during juice processing may possess potent beneficial role to use for our health promotion. In fruit residue and kernels parts from eight different cultivars, determination of cyaniding-3-O-glucoside (C3G) levels using HPLC (high performance liquid chromatography), various functional assays including antioxidant, and antifungal assays in vitro, cytotoxic assays and also antifatigue assays in vivo using reducing testes, rectangular cantilever method, small microtiter-plate method and mice loaded swimming test, respectively. The results of the HPLC method showed the extracts of residue were rich in cyaniding-3-O-glucoside, especially in *Myricarubra* Sieb. Et Zucc. cv. Biqi (212.8 mg·g⁻¹ from Ninghai and 185.7 mg·g⁻¹ from Yuyao). The results of the activity experiments showed the TE values of the extract from Biqi were between 334 and 506 mmol·g⁻¹ detected by different methods. The duration of loaded swimming to exhaust prolonged from 614±158s to 1012±205s. The MIC (minimal inhibitory concentration) values of different species of kernels extracts against 8 different fungi ranged from 0.5000 to 0.0625 mg·mL⁻¹. The LC50 were from 49-316 µg·mL⁻¹. In conclusion, the waste part of bayberry produced during juice processing, the residue and kernels, is of potential application value. However, further study is required.

Keywords: anthocyanin, antioxidant, antifungal, cytotoxic, antifatigue

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1. Introduction

The Myricaceae plant *Myricarubra* Sieb. Et Zucc., which has a long cultivating history and high yield in China, is one of the most important edible fruits with a high anthocyanin content. Many researchers proved that anthocyanins are very helpful in preventing glycaemia [1-5] and gastric cancer [6,7,8]. However, not every anthocyanin has anticancer or antidiabetic biological activity. Cyanidin-3-O-glucoside (C3G) is one of the most common anthocyanins. Previous research [9,10,11] proved that C3G had obvious effects on treating glycaemia and gastric cancer. It has been reported that *Myricarubra* juice has a high content of C3G [12,13]. However, it is very difficult to develop and utilize *Myricarubra* because of its high sugar content. The exploitation of *Myricarubra* is seriously impaired by the waste of hundreds and thousands of tons of its residues and kernels, the disposal of which is very difficult [14]. Furthermore, because of the large varieties of *Myricarubra*, it is common for us to ignore the best varieties in food industry. In this paper the extracts of 8 cultivated varieties of *Myricarubra* were prepared and analyzed by HPLC (high performance liquid

chromatography). Meanwhile, the antioxidant, antifungal, cytotoxic, and antifatigue activities of the extracts were investigated. These results will help us to seek out the best varieties of *Myricarubra* and also help us to understand the potential value of its two waste materials.

2. Materials and Methods

2.1. Materials and Chemicals

Myricarubra fruit of 8 cultivars were obtained in Zhejiang Province, China in June, 2011. *Myricarubra* Sieb. Et Zucc.cv. Biqi¹, *Myricarubra* Sieb. Et Zucc.cv. Tanmei and *Myricarubra* Sieb. Et Zucc.cv. Shuijing were from Ninghai, *Myricarubra* Sieb. Et Zucc.cv. Biqi² was from Yuyao, *Myricarubra* Sieb. Et Zucc.cv. Dongkui from Taizhou, *Myricarubra* Sieb. Et Zucc.cv. Ding-ao from Wenzhou, *Myricarubra* Sieb. Et Zucc.cv. Wandao from Zhoushan, and wild species *Myricarubra* Sieb. Et Zucc. was from Lin'an, respectively. Fruit of each cultivar was stored at -20°C until analysis. Fungal strains, namely *Fusariumgraminearum* Schwabe, *Fusariumgraminearum*, *Exerohilumturcicum* Pass, *Rhizoctoniasolani* Kühn, *Botrytis cinerea*, *Valsamali*, *Sclerotiniasclerotiorum* and

Fusariumoxysporum f. sp. *Vasinfestum* are provided by microbiological lab of Zhejiang Agriculture and Forestry University. Male mice were purchased from Institute of Materia Medica, Zhejiang Academy of Medical Sciences, China. *Artemiasalina* was purchased from Fengnian Aquaculture Co., Ltd Tianjin, China.

Standards of C3G, rutin, gallate acid were purchased from the National Assaying Institute for the Pharmaceutical and Biological Products. DPPH (1, 1-diphenyl-2-picrylhydrazyl); TPTZ (2, 4, 6-Tris (2-pyridyl)-s-triazine); Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Methanol was chromatographically pure and the other organic solvents and chemicals used in this study were of analytical pure.

2.2. Preparation of Purified Anthocyanins Extracts (PAEs)

Approximately 500 g of *Myricarubra* fruit of each cultivar was denucleated and mashed, respectively. The squeezed residue was extracted twice with the ratio of 1:2 (v/v) pH 3 by 50% ethanol solutions using ultrasonic extraction for 1 h and Rotary-evaporated method under vacuum at 60°C until the ethanol was evaporated completely. Then it was passed through resin columns (Diaion, HP2 MGL, Mitsubishi Co of Japan, 6 cm × 30 cm) depending on the adsorbent capability of each resin. C3G was adsorbed onto the column, and sugar, acids and other water-soluble compounds were eluted with more than 2 BV of distilled water until the wash water was clear [15]. The adsorbed material was then eluted with acidified 60% ethanol (v/v, pH 3). The eluent was concentrated on a rotary evaporator under 60°C until the pigment powder was prepared and stored in a cool dry place before analysis. The crystal *Myricarubra* had the color of white and contained no anthocyanidin, so we didn't get its extracts.

2.3. Preparation of Extracts From Kernels (Eks)

The 8 bayberry kernels of cleaned and dried varieties were screened through mesh size of 60 after shattered, then stored in a dry place. Weigh accurately 1.00 g powder, then added 30 mL 70% ethyl alcohol. After 2 times of ultrasonic extraction (1 h each time), the extraction solution was amalgamated and concentrated under reduced pressure at 60°C, and then brought to 10 mL with 70% chromatographically pure ethyl. The solution was kept in cold storage before analysis.

2.4. HPLC Analysis

Contents of C3Gs were analyzed with a Waters 2695 high performance liquid chromatograph linked simultaneously to a Waters 2996 photodiode array detector. All samples were filtered through a 0.45 µm Millipore membrane filter before injection. A 10 µL aliquot was separated by a SunFire-C18 (Waters, USA) column (250 mm × 4.6 mm, 5 µm) at 30°C. The mobile phase consisted of solvent A (2% hydrochloric acid in methyl alcohol) and solvent B (water, methyl alcohol, acetonitrile, acetic acid = 160:90:90:40, v/v/v/v). Using

isocratic elution with A:B = 93:7 with the flow rate 1.0 mL·min⁻¹, C3G was detected at 530 nm.

2.5. DPPH Assaying Method

The DPPH assay was utilized by 96-well plates with some modifications [16]. The absorbance in this study was measured by automatic microplate reader (Infinite M 200 Pro, Tecan). The PAEs of 1.00 mg from 7 different cultivars were dissolved with 10 mL absolute ethyl alcohol, respectively. 5, 10, 15, 20, 25 µL of PAEs and EKs solution were added to 96-well microplates and then 195, 190, 185, 180, 175 µL absolute ethyl alcohol was added to each well respectively. 50 µL DPPH were added to the microplates and incubated at 25°C for 30 minutes, then the ultraviolet absorption (As) was determined at 517 nm. The antioxidant activity (%) of radical-scavengers was calculated as $(As - Ar / Ao) \times 100\%$, where As and Ar are the absorbance of the sample and control, respectively, at 517 nm. Trolox was used as the standard. Results were expressed as TE (mmol Trolox equivalents) per g dry weight.

2.6. FRAP (Ferric Reducing Antioxidant Power) Assaying Method

The reducing abilities were measured by DU 800 visible ultraviolet spectrophotometer (Beckman Coulter Inc.) [17]. The FRAP assay was carried out, where the FRAP reagent was made from 10 mmol·L⁻¹ TPTZ and 20 mmol·L⁻¹ ferric chloride in 0.1 mol·L⁻¹ sodium acetate buffer (1:1:10, v/v/v, pH 3.6). 10 min after the combination of 150 µL FRAP with 50 µL solution (0.2 mg·mL⁻¹, absolute ethyl alcohol), the absorbance at 593 nm was measured, where the standard curve was constructed using a linear concentration of Trolox. Results were expressed as TE per g dry weight.

2.7. TRP (Total Reducing Power) Assay Method

The TRPs were determined according to the method procedure described by Oyaizu [18]. Each 500 µL solution (0.2 mg·mL⁻¹, absolute ethyl alcohol), sodium phosphate buffer (2.5 mL, 0.2 mol·L⁻¹, pH 6.6) and potassium ferricyanide (2.5 mL, 10 mg·mL⁻¹) were mixed and incubated at 50°C for 30 min. Trichloroacetic acid (2.5 mL, 100 mg·mL⁻¹) was added to the mixture and centrifuged at 4000 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% ferric chloride (0.5 mL), and then its absorbance was measured at 700 nm against a blank. Trolox was used as a control and the results were expressed as TE per g dry weight.

2.8. Antifungal Experiment and MIC (Minimal Inhibitory Concentration) Measurement

This research improved the method of Zhang, by evaluating antifungal activity of PAEs and EKs [19]. Diluted EKs and PAEs solution with 70% ethyl-alcohol in multiples to sample solutions with concentrations as 1.000, 0.500, 0.250, 0.125, 0.0625 and 0.03125 mg·mL⁻¹. 5 mL well prepared PDA (potato dextrose agar) culture

medium was added to test tubes, and was made to slant culture medium after 20 min sterilization at 121°C, then 500 µL sample solutions of different concentrations were added to the cooling culture medium. After sample solvent was volatilized, 8 kinds of fungi were inoculated with streak-plating method. The inoculated medium was sealed and cultivated at 25°C, then observed the growing state of Hyphae after 48 h. 3 groups of parallel experiments were carried out for each sample. The minimal sample concentration was expressed as MIC.

2.9. Brine Shrimp Lethality Assay

The brine shrimp toxicity was assayed by modified small microtiter-plate method using brine shrimp *Artemiasalina* as a test organism [20]. 0.5, 1, 2, 4, 8, 10 µL PAEs solution (0.10 mg·mL⁻¹ of the distilled water) and EKs solution (0.10 g·mL⁻¹ of the distilled water) were added into the microtiter-plate, and then a suspension of *Artemiasalina* containing 15-20 organisms (199.5, 199, 198, 196, 192, 190 µL) was added to each well, respectively. Distilled water in the same volumes was used to substitute for the sample as the negative controls, respectively. The number of organisms in each well was recorded in time and shielded from light. Being incubated at 28 ± 0.5°C for 24 h, the number was recorded again. The corrected mortality rate was calculated according to the following formula: Corrected mortality rate (%) = [(T-C)/(1-C)] × 100%, where T was the mortality rate of the treatment and C was the mortality rate of the negative control. The results were expressed as LC₅₀, the concentration which corrected mortality rate was 50%.

2.10. In Vivo Antifatigue Effect

This article estimated the *in vivo* antifatigue activity with mice loaded swimming experiment [21]. Several experimental mice with 20 ± 2 g weights which had adapted to the lab-environment for one week were randomly assigned to 3 experimental groups and each blank group consisting of dozens of mice. *In vivo* antifatigue effect of EKs were negative in preliminary experiment and PAEs from Biqu¹ were prepared into the solutions with 3 concentrations (high concentration, 0.3320 mg·mL⁻¹; middle concentration, 0.2656 mg·mL⁻¹; low concentration, 0.1992 mg·mL⁻¹) with sodium carboxy methyl cellulose, and then the mixed solutions were tested with sodium carboxy methyl cellulose as control sample. The experimental mice were lavaged and weighted with 0.4 mL sample every 24 h, and when the mice weights approached to 30 g, the lavage dosage was increased to 0.6 mL. By the time the intragastric administration was carried out for 30 d, the antifatigue ability of mice was determined by loaded swimming. After treated with PAEs from Biqu¹ for 30 min, the experimental mice were allowed to swim in a swimming tank (volume of water, 50 cm × 35 cm × 40 cm; water temperature, 25 ± 0.5°C) with a 5% mice weight lead sheath attached to the root of tails, and the time of mice exhaustion to death was recorded by stopwatch from the start of the swimming until the heads of mice were drowned beneath water for 10 s without moving.

2.11. Statistical Analysis

Each experiment was carried out in triplicate. Data were analyzed using the One-way ANOVA of SPSS.19 (Statistical Package for Social Science Institute, Cary, N.C.). Significant differences (p < 0.05) between mean values were determined using Duncan's multiple-range tests.

3. Results and Discussion

3.1. Determination of C3G Content

HPLC results show that, apart from the EKs and Shuijing (crystal *Myricarubra*, which were free of anthocyanin), 7 kinds of anthocyanin bayberry extracts all displayed one main chromatography peak (taking up about 95% of the total chromatographic peak area) with the detection wavelength of 530 nm, while other peaks took up rather small area. Main peak was determined to be C3G after a peak time comparison analysis with standard substance, and the result was similar to the previous research [12] which had not studied the content difference among cultivated varieties. From Figure 1, we found that among the PAEs from 7 cultivated varieties, Biqu had the largest content of C3G (212.8 mg·g⁻¹ from Ninghai, and 185.7 mg·g⁻¹ from Yuyao) is 250 times than that of in juice [22].

Our research purified the anthocyanins from 8 cultivated varieties of *Myricarubra* with macroporous resin, and then PAEs free of carbohydrates were obtained, which were rich in property-stable C3G. If applied in food additives, PAEs may avoid the loss of anthocyanins during processing and storage of bayberry, and the consumption-suitable range may be extended. PAEs from Biqu had the highest content of C3G, and the extraction rate of Biqu was found to be the highest of the 8 cultivated varieties. Considering these two factors, Biqu was more suitable for being exploited as food additives and health-care products than other varieties.

3.2. Antioxidant Activity Evaluation

Table 1. The TE (mmol·g⁻¹) value of PAEs (purified anthocyanins extracts) and the relativity analysis between C3G contents and antioxidant capacity of PAEs

	DPPH	FRAP	TRP
Dongkui	275±19c	352±25b	418±13b
Ding-ao	212±9.6d	294±8.5c	333±12c
Wandao	173±2.3de	240±3.5d	276±13d
Biqu ¹	451±63a	471±33a	506±29a
Biqu ²	334±8.0b	361±17b	444±13b
Tanmei	159±19e	159±17e	245±9.6e
Wild species	128±3.1e	133±30e	171±7.8f
R ²	0.888	0.961	0.967

All the measurements were taken in triplicate and the results are expressed as mean values ± standard deviations. Values followed by different letters within the same row represent significant differences at P < 0.05.

Except crystal *Myricarubra*, the antioxidant activities of 7 PAEs and 8 EKs were detected by 3 different methods at the same time. Antioxidant activities of EKs were not found. And the result shown in Table 1 and Figure 2, was that 7 kinds of PAEs all had great free radical scavenging capacity and reducing capacity with good data consistency in the three methods. Compared with other varieties, PAEs from Biqu showed strongest scavenging activity of DPPH radicals and reducing

capacity. Being both from Ninghai and Yuyao, the TE values were between 334 and 506 $\text{mmol}\cdot\text{g}^{-1}$ detected different methods, and particularly, Biqu from Ninghai had TE value of more than 450 $\text{mmol}\cdot\text{g}^{-1}$, by 3 Dongkui (275-418 $\text{mmol}\cdot\text{g}^{-1}$), Ding-ao (212-333 $\text{mmol}\cdot\text{g}^{-1}$) and Wandao (173-276 $\text{mmol}\cdot\text{g}^{-1}$). Tanmei (159-245 $\text{mmol}\cdot\text{g}^{-1}$) was the lowest among the 7 cultivated varieties, and the wild species was lower than all 7 cultivated varieties. Still, all of the PAEs showed much stronger antioxidant capacity than fresh fruits in previous researches. There might be

two chief reasons: First, samples were processed by different methods. In this article the experimental materials were purified PAEs which contained more C3G than fresh fruits with stable properties. The analysis showed that the antioxidant capacity correlated closely with C3G contents, and the correlation coefficients (R^2) of 3 methods were 0.888, 0.961 and 0.967, respectively, which indicated that C3G played an important role in antioxidant activity of PAEs from different bayberry varieties.

Figure 1. HPLC chromatogram (at 530 nm) of a = C3G standard, b= C3G in Biqui¹, c=C3G in different PAEs (purified anthocyanins extracts). (Chromatographic peaks from left to right in figure c indicated C3G contents from Wandao, Biqui², Ding-ao, Biqui¹, Dongkui, wild species, Tanmei, respectively.)

Figure 2. Contents of C3G and antioxidant capacity (TE) of PAEs. Different letters mean significant differences ($p < 0.05$)

3.3. Antifungal Activity

We evaluated the antifungal capability of PAEs and EKs with slant-face-tube method, and the results (Table 2) showed that EKs exhibited effective antifungal capability against sample fungus except *Botrytis cinerea* and *Fusarium oxysporum* f. sp. *Vasinfestum* with MIC values ranging from 0.5000 to 0.0625 mg·mL⁻¹. Thus it could be seen that *Myricarubra* kernel was a good source of low-priced botanical fungicide. At the same time, restraint activities of PAEs were not found.

3.4. Cytotoxic Activity

In previous brine shrimp lethality assay, estimating the cytotoxicity of compounds, monomers with LC₅₀ less than 100 µg·mL⁻¹ and crude extracts with LC₅₀ less than 500 µg·mL⁻¹ were deemed to be cytotoxic. As shown in Table 3, PAEs from bayberries distributed their LC₅₀ from 500-1000 µg·mL⁻¹, which showed that there was little or none

antifungal activity or cytotoxicity in the extracts of *Myricarubra* and anthocyanidin. These research results were strong evidence of the feasibility and safety for these extracts to be developed as food additives and health-care food.

On the contrary, the results showed that a strong cytotoxicity was potent in EKs and no cytotoxicity in PAEs. LC₅₀ of EKs and PAEs ranged from tens to hundreds. The LC₅₀ of wild species of the bayberry kernels with highest potent cytotoxic activity was equivalent to numerous potent cytotoxic monomers in previous researches. Moreover, previous researches confirmed that cytotoxic substances could specifically inhibit tumor-cell proliferation, which indicated a promising future as it is cheap and easy to obtain, and it solved the waste-disposal problem in bayberry food industry. However, no obvious correlation between EKs' cytotoxicity and flavonoids or phenolic acids compounds was found, so the mechanisms of EKs' cytotoxicity should be further studied.

Table 2. Antifungal activities and MIC (mg·mL⁻¹) values of EKs

	<i>Fusarium graminearum</i> Schwabe	<i>Fusarium graminearum</i>	<i>Exerohilum turcicum</i> Pass	<i>Rhizoctonia solani</i> Kühn	<i>Botrytis cinerea</i>	<i>Valsa mali</i>	<i>Sclerotinia sclerotiorum</i>	<i>Fusarium oxysporum</i> f. sp. <i>vasinfestum</i>
Dongkui	0.1250	0.1250	0.2500	0.5000	a	0.2500	0.1250	a
Ding-ao	0.2500	0.1250	0.5000	0.2500	a	0.2500	0.2500	a
Wandao	0.2500	0.2500	0.0625	0.1250	a	0.2500	0.2500	0.1250
Biqi ¹	0.1250	0.2500	0.2500	0.2500	a	0.2500	0.2500	0.5000
Biqi ²	0.2500	0.2500	0.2500	0.5000	a	0.5000	0.2500	a
Tanmei	0.2500	0.2500	0.2500	0.2500	a	0.5000	0.5000	0.5000
Shuijing	0.2500	0.5000	0.5000	0.2500	a	0.2500	0.1250	0.2500
Wild species	0.0625	0.0625	0.2500	0.1250	0.5000	0.5000	0.1250	0.2500

^a means mycelial growth were all observed in three tubes

Table 3. The cytotoxicity of EKs (extracts from kernels) and PAEs

EKs	LC ₅₀ (µL·mL ⁻¹)	PAEs	LC ₅₀ (µL·mL ⁻¹)
Dongkui	180	Dongkui	759
Ding-ao	128	Ding-ao	1038
Wandao	58	Wandao	521
Biqi ¹	134	Biqi ¹	769
Biqi ²	195	Biqi ²	670
Tanmei	72	Tanmei	463
Shuijing	316	Shuijing	ND
Wild species	49	Wild species	803

ND: Not Detect.

3.5. Antifatigue Activity

Recent research showed that anthocyanins of mulberry juice purification which contained two kinds of anthocyanin and one of them was C3G, possessed an antifatigue effect and an increased endurance capacity [23]. Therefore, in this paper, the loaded swimming experiment, the most direct, objective criterion for reflecting physical endurance, was designed to authenticate whether PAEs had such an activity as well,

and whether C3G played a major role in it. The results (Figure 3) of the testing indicated that PAEs helped to prolong the time of mice-loaded swimming: The higher dose groups with Biqui¹ PAEs could significantly prolong the time of mice-loaded swimming, while the low and medium-dosage groups could also increase the swimming time to a certain extent. Thus it can be seen that: Biquianthocyanins have vast development future when used as the food additives.

Figure 3. Effect of PAEs of Biqui¹ on the swimming time in mice. The error bars represent one standard deviation. Different letters indicate significant differences between groups ($P < 0.05$)

4. Conclusion

As the most important two parts of the industrial waste from processing *Myricarubra*, the residue and kernels have huge value and potential commercial application. The residue is rich in anthocyanins and C3G, and it has strong effects on antioxidant and fatigue resistance without any sugar and toxicity. It could be used as food additives and dietary supplement. On the other hand, kernels with high cytotoxic and antifungal activities bring health and medical value. This industrial waste deserves to be recycled and further developed

List of Abbreviations

C3G: cyanidin-3-O-glucoside
 DPPH: 1, 1-diphenyl-2-picrylhydrazyl
 TE: mmol Trolox equivalents
 FRAP: ferric reducing antioxidant power
 TPTZ: 2, 4, 6-tris (2-pyridyl)-s-triazine
 PDA: potato dextrose agar
 PAEs: purified anthocyanins extracts
 EKs: extracts from kernel
 TRP: total reducing power
 MIC: minimal inhibitory concentration

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