JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Assessment of the Antibacterial Activity and the Antidiarrheal Function of Flavonoids from Bayberry Fruit

Wei Rong Yao,* He Ya Wang, Shi Tao Wang, Shi Lei Sun, Jie Zhou, and Yun Yun Luan

State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi, Jiangsu 214122, China

Supporting Information

ABSTRACT: Chinese bayberry fruits are used as a folk medicine to cure diarrhea. However, the active compounds have not yet been reported. We found that bayberry fruit extract showed significant antibacterial activity against *Salmonella*, *Listeria*, and *Shigella*, and the minimal inhibitory concentration (MIC) ranged from 2.07 to 8.28 mg/mL. Positive relationships were found between the antibacterial activity and the total polyphenol ($r = 0.88$) and flavonoid contents ($r = 0.92$) of samples using different extraction times. The active compounds showed green or blue fluorescence under UV light using the bioautography method and were purified using a polyamide column. The fraction F1 with the most activity was comprised of flavonoids, which included cyanidin-3-*O*-glucoside, myricetin deoxyhexoside, quercetin-3-*O*-glucoside, and quercetin deoxyhexoside, and it also possessed an antidiarrheal activity ($p < 0.10$) at 80 mg/kg in mice. These findings provide scientific evidence for the antidiarrheal function of bayberry.

KEYWORDS: Bayberry, antibacterial activity, antidiarrheal, flavonoids

INTRODUCTION

Myrica rubra Sieb. et Zucc. is a tree that belongs to the Myricaceae family, of the genus *Myrica*, and is widely distributed in China, Japan, Korea, and the Philippines. The fruit of *M. rubra* Sieb. et Zucc. is named bayberry, and China has an annual production of approximately 300000 tons. The fruit is consumed fresh or used for juice, jams, or winemaking, but it is also prepared as an agent to cure diarrhea in traditional Chinese medicine. For more than 2500 years, bayberry, immersed in a distilled spirit, has been used to cure diarrhea; however, it continues to be used in domestic medicine because its antidiarrheal properties have not been widely developed due to a lack of scientific evidence. Except for information on the prolonged shelf life of the fresh fruit, most research has focused on bayberry juice production,[1,2] the by-products of the pomace and kernel, and their antioxidant activities.[3,4] In addition, antibacterial activity of the leaf of *M. rubra* Sieb. et Zucc. has been reported.[5] In the book of Chinese herbal medicine, Compendium of Materia Medica,[6] the medical application of the leaf cures enteritis, hemostasia, and arthralgia, and the root and stem cure scalding, carbuncle, and bellyache. However, these reports are not sufficient to illustrate the mechanism of the antidiarrheal function of bayberries.

Many reports have been focused on the relationship between the antidiarrheal function and the antibacterial activity of plants extracts used in traditional medicine, such as the extracts of 26 medicinal plants from Mexico,[7] 23 medicinal plants from South Africa,[8] *Azadirachta indica* from India,[9] and *Byrsonima fagifolia* from Brazil.[10] These studies have validated that a portion of the antidiarrheal action is due to their antibacterial properties. The leaves of *M. rubra* Sieb. et Zucc. possess antibacterial activity, and phenols have been identified as the active compounds.[11] In addition, a high content of phenolic compounds is found in the bayberry fruit,[4] the juice product,[2] and even the byproducts of the pomace.[3,12] Moreover, many other kinds of berries, such as cloudberry and raspberry, show significant antibacterial activity against food-borne pathogens and have phenols as their main active compounds.[13,14] Therefore, further research on the bioassay-directed fractionation of the active crude extracts from bayberry is needed to isolate and identify the compounds responsible for the antibacterial activity.

To further understand the bioactivity of bayberry fruit and its active compounds, this study was focused on the in vitro antibacterial activity and the in vivo antidiarrheal function of the bayberry extract; the flavonoids with bioactivity were traced, analyzed quantitatively, and characterized.

MATERIALS AND METHODS

Chemicals. The chemical standards used were purchased from Sigma (United States), including quercetin-3-*O*-glucoside, the Folin−Ciocalteu phenolic reagent, gallic acid, polyamide, rutin, and 2,3,5-triphenyltetrazolium chloride (tetrazolium red). Cyanidin-3-*O*-glucoside was obtained from the Yuancheng Co. (Wuhan, China). Berberine was obtained from the Shanghai Medicine Co. (Shanghai, China). Mueller−Hinton broth was obtained from Oxoid (Wesel, Germany). Nutrient agar was obtained from the Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The other solvents and reagents were analytical grade and obtained from the Shanghai Chemical Reagent Co. (Shanghai, China).

Preparation of the Crude Extract. To ensure that the bayberry fruit used in our experiment was of the same cultivar, the bayberry (cultivar Biqi) was purchased from a commercial market in Wuxi City, Jiangsu Province, China, in June 2007. Portions of about 100 g (4−6 granules of fruit) were packed in polyethylene bags and subsequently

Received: January 21, 2011
Accepted: April 6, 2011
Revised: April 2, 2011
Published: April 06, 2011

Table 1. Antibacterial Activities of the Crude Extract from Bayberry against 14 Food-Borne Pathogens

strain	diameters of inhibition zone (mm ± SD)	MIC (mg/mL)[a]
Salmonella paratyphi A CMCC[b] 50093 (−)[c]	25.9 ± 1.2	2.07
Salmonella enteritidis ATCC[d] 13076 (−)	20.6 ± 1.9	8.28
Salmonella typhi CMCC 50013 (−)	13.9 ± 1.7	8.28
Salmonella CNAS[e] (−)	16.1 ± 3.2	8.28
Escherichia coli ATCC 8099 (−)	16.6 ± 0.6	8.28
Shigella dysenteriae CMCC 51334 (−)	19.9 ± 1.9	4.14
Shigella dysenteriae CMCC 51573-10 (−)	22.3 ± 1.2	4.14
Pseudomonas aeruginosa ATCC 14149 (−)	16.4 ± 0.6	4.14
Vibrio parahemolyticus ATCC 17803 (−)	22.0 ± 1.0	4.14
Staphylococcus aureus ATCC 6538 (+)[f]	21.2 ± 0.9	4.14
Streptococcus hemolyc CMCC 32210 (+)	21.3 ± 1.3	4.14
Listeria monocytogenes ATCC 35152 (+)	17.1 ± 0.8	2.07
Listeria welshimeri ATCC 35897 (+)	16.0 ± 2.5	2.07
Listeria innocua ATCC 33090 (+)	21.6 ± 1. 8	2.07

[a] The values of MIC were expressed by the weight of the crude extract in the volume of medium. [b] CMCC: National Center for Medical Culture Collection, China. [c] Gram-negative strain. [d] ATCC: American Type Culture Collection (United States). [e] *Salmonella* CNAS was isolated from a frozen meat product by Zhangjiagang Entry-Exit Inspection & Quarantine Bureau. [f] Gram-positive strain.

frozen at −18 °C. For the extraction, the frozen bayberry fruit was homogenized in a mortar with ethanol (1:2.5, w/v), and the mixtures were shaken at 200 rpm for 24 h at 50 °C in a water bath shaker. The extract was centrifuged at 4000 rpm for 10 min, and the supernatant was lyophilized to dryness. This powder of crude extract was weighed (the yield was 8.28 g of 100 g fruit) and termed "BPCE" in our research. The BPCE was redissolved in 5.0 mL of water before the determination of the phenol content and the bioactivity detection.

Phenol Detection. To further understand the phenol compounds in bayberry extracts, the contents of total polyphenols, total flavonoids, and tannins were analyzed. The amount of total polyphenols in the crude extract was determined according to the Folin−Ciocalteu procedure.[15] Aliquots of appropriately diluted or standard solutions (0.2 mL, two replicates) were placed in test tubes, and 1.0 mL of 0.2 mol/L Folin−Ciocalteu reagent and 0.8 mL of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 60 min. The absorption at 765 nm was measured (Shimadzu UV-2450, Japan), and the total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per kilogram BPCE.

The total flavonoid content was determined by a colorimetric method.[4] Aliquots (0.5 mL) of appropriately diluted or standard solutions were pipetted into 15 mL polypropylene conical tubes containing 2 mL of double-distilled H_2O and mixed with 0.15 mL of 5% $NaNO_2$. After 6 min, 0.15 mL of 10% $AlCl_3 \cdot 6H_2O$ solution was added. The mixture was allowed to stand for another 5 min, and then, 1 mL of 1 mol/L NaOH was added. The reaction solution was well mixed and incubated for 15 min, and the absorbance was determined at 415 nm. The total flavonoid content was calculated using the standard rutin curve and expressed as milligrams of rutin per kilograms BPCE.

The content of tannins in the extracts was determined using a titrimetric method with potassium permanganate solution and indigo-carmine.[16] The appropriately diluted solution (5 mL) was mixed with 10 mL of 0.1% solution digo-carmine and 100 mL of distilled water; the mixture was then titrated with 0.05 mol/L potassium permanganate solution to a gold-yellow color, and the consumption volume was recorded. A control titration (without extract) was used as a blank. The contents of tannins were expressed as mg/kg of BPCE and determined using the equation below:

$$\text{the content of tannins} = 0.05 \times (V_{\text{sample}} - V_{\text{control}}) \times 0.004157 \times 100/(W \times 0.1000)$$

The consumption of 1.00 mL of potassium permanganate solution was defined as equal to 0.004157 g of tannins, and "W" represented the kilograms of BPCE that were added to the reaction mixture.

Preparation of Bacteria. All of the bacteria were grown in nutrient agar except *Streptococcus hemolyticus* CMCC 32210, which was cultured in cation-adjusted Mueller−Hinton broth plus 5% lysed horse blood. The microbial strains are listed in Table 1. Single colonies of the selected strains of bacteria were preinoculated in 10 mL of sterile nutrient broth and incubated at 37 °C for 8 h; 1 mL of the culture was then added to 100 mL of broth and incubated at 37 °C for 24 h. The culture was diluted to a suitable concentration and used for the antibacterial tests.

In Vitro Antibacterial Test. The cylinder diffusion method was used to detect the diameter of the inhibition zone. The culture medium was premixed with bacteria to a cell concentration of approximately 10^6 cfu/mL at approximately 48 °C, and approximately 15 mL of culture medium was then immediately poured into each Petri dish (9 cm in diameter) before the medium solidified. Three evenly placed wells (6 mm in diameter) were put onto the surface of the solidified medium, and 200 μL of the extract was added into the wells using a pipet (Finnpipette, France). The plates were incubated at 23 °C for 1 h to facilitate diffusion and then incubated at 35 °C for 24 h.[17] The diameter of the inhibition zone was measured to an accuracy of 0.1 mm, and the effect was calculated as the mean of six tests. Water (200 μL) and polymyxin B sulfate (90 μg in 100 μL) were used as negative and positive controls, respectively.

The 2-fold dilution method was used to determine the minimal inhibitory concentration (MIC). A 2-fold dilution series of BPCE in nutrient broth was prepared to obtain final concentrations of 16.56, 8.28, 4.14, 2.07, 1.04, or 0.52 mg/mL. Approximately 10^6 CFU/mL of each bacterial culture was mixed into the broth, and the lowest concentration without any colony growth was recorded as the MIC value.[18] The experiments were conducted in triplicate.

Bioautographic Investigations. To trace the antibacterial activity, bioautography analysis was used. The sample of BPCE was first pretreated by solid-phase extraction (SPE). A 5 mL solution of BPCE was loaded onto an SPE column (Supelclean C-18, 500 mg, 6 mL column volume), which had been equilibrated with ethanol (5 mL) and water (5 mL). The sample on the SPE column was washed successively with chloroform, petroleum ether, and ethyl ether (5 mL of each solvent), and then, ethyl acetate and water (10 mL of each solvent) were used to elute the active compounds sequentially. The eluates were combined and concentrated under reduced pressure to dryness to obtain the partially purified sample.

For the thin-layer chromatography (TLC) separation, 10 mg of the partially purified sample was redissolved in distilled water, loaded onto TLC plates, dried, and developed in the mobile phases of acetone−water (7:5). After the solvent migrated to a position of 2 cm from the top of the plates, the plates were placed in a stream of air for at least 24 h to evaporate the solvent thoroughly and then sprayed with a culture of *S. paratyphi* CMCC 50093 at a concentration of 10^8 CFU/mL until completely moist. The moist plates were incubated at 37 °C in a humidified chamber for 2 h and then sprayed with 0.5 mg/mL of 2,3,5-triphenyltetrazolium chloride (tetrazolium red) and incubated for another 12−24 h. The clear zones against a red-colored background were determined.[19]

Fractionation of Phenols from the Crude Extract with a Polyamide Column. A polyamide column was used to fractionate the phenols present in the bayberry extract. The partially purified sample from the SPE was loaded on an open column overlaid with polyamide (2.5 cm × 30 cm) and eluted with 10, 40, or 60% at 0.8 mL/min. On the basis of the absorbance at 254 nm, fractions were collected using a fraction collector (5 min per tube). The solvent was then removed by rotary evaporation followed by freeze drying. Before the antibacterial activity was tested, the fractions were dissolved in distilled water.

Identification of Flavonoids by Ultraperformance Liquid Chromatographic−Electrospray Mass Spectrometry (UPLC-ESI-MS). To identify the phenol compounds with antibacterial activity, UPLC-ESI-MS was used. Before analysis, the samples were scanned at 190−700 nm (Unico UV-4802, Unico, NJ) to ensure the measurement of the phenolic compounds. The UPLC-ESI-MS was performed using an Acquity UPLC-MS (Waters Corp., United States) equipped with a photodiode array detector (DAD). The data were collected and processed using Waters MassLynx_V4_RC9 software. The column was a BEH C-18 (2.1 mm × 50 mm, 1.7 μm particles), and the mobile phase consisted of 0.1% formic acid, 5% acetonitrile, and 95% H_2O (V/V) (A) and acetonitrile containing 0.1% formic acid (B). A stepwise linear gradient was programmed at 99:1 (A/B, V/V) for 2 min, changed to 98:2 over 5 min, and changed to 60:40 over 1 min, followed by 10:90 over 5 min to wash the column. The injection volume was 5 μL, and the detection wavelength was 254 nm (each fraction had an absorbance at this wavelength). The column temperature was 40 °C.

The mass spectra were achieved by electrospray ionization in the positive mode. Used were the following ion optics: capillary, 3.88 kV; cone, 25 V; and extractor, 5 V. The source block temperature was 120 °C, and the desolvation temperature was 300 °C. The electrospray probe flow was adjusted to 70 mL/min. Continuous mass spectra were recorded over the range m/z 100−800 with a 1 s scan time and an interscan delay of 0.1 s (s). Standards of cyanidin-3-*O*-glucoside and quercetin-3-*O*-glucoside were also detected.

Antidiarrheal Function in Mice with Diarrhea Induced by *Salmonella*. To study the in vivo antidiarrheal activity of the bayberry extract and its further fraction, an animal test was performed. These experiments were performed with Swiss albino mice of both sexes, weighing 18−25 g, obtained from the experimental animal center of Fudan University.

The mice were administered 0.5 mL of *S. paratyphi* A CMCC 50093 (a total of about 10^9 CFU) orally. After 1 h, the animals with diarrhea were divided into negative control, positive control, and test groups with 10 mice in each group (M:F = 1:1). The negative control group was orally administered water at a dose of 40 mg/kg, whereas the positive control group was orally administered berberine at a dose of 40 mg/kg. The test groups were administered 100 mg/kg BPCE and fraction F1 at doses of 40, 80, or 120 mg/kg orally. Each animal was placed in an individual cage, the floor of which was lined with blotting paper. The floor lining was changed every hour. During the 6 h observation, the number of total and wet feces excreted by the animals was recorded, and the percent reduction in the number of wet feces in the treated group was obtained by comparison with the negative control animals.

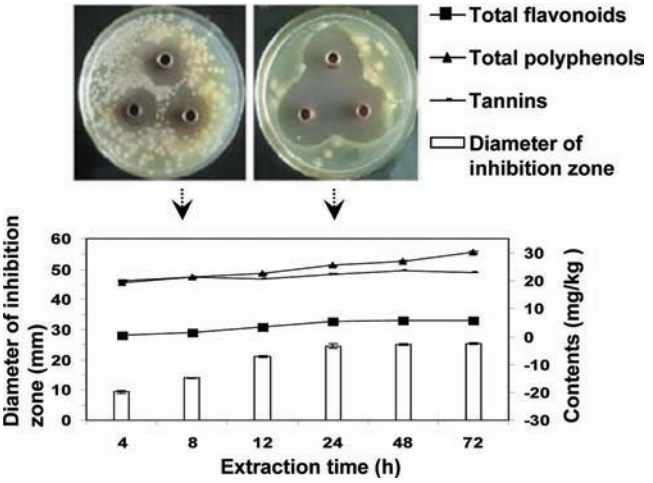

Figure 1. Antibacterial activity and the phenol content of the ethanol extract. The lower graph shows the effects of the ethanol treatment time on the diameter of the inhibition zone and the total polyphenols, flavonoids, and tannin content. The upper panel shows the photos of the inhibition zone of the ethanol crude extract, after 8 and 24 h, against *S. paratyphi* A CMCC 50093. Extract (200 μL) from different ethanol−extraction times was added into the hole of the cylinder. The diameter around the cylinder (no growth of colony) was recorded as the inhibition effect, which was measured to an accuracy of 0.1 mm as the mean of six tests. Ethanol (200 μL) was used as a negative control (no observed inhibition effect).

Statistical Analysis. The statistical analysis in this research was performed by SPSS software (SPSS 16.0 for Windows, SPSS Inc., United States). The data were processed using a one-way variance analysis (ANOVA). Differences of $p < 0.10$ were considered significant in the antidiarrheal function experiments. In the other experiments, differences of $p < 0.05$ were considered to be significant.

RESULTS

Antibacterial Activity of Bayberry Crude Extracts. Our results showed that bayberry extract had a satisfactory antibacterial activity on all of the tested food-borne pathogens, including both Gram-positive bacteria and Gram-negative bacteria (Table 1). The MIC values ranged from 2.07 to 8.28 mg/mL, which was the same level of the MIC values from the fruit (at the level of mg/mL).[20]

Effect of Extraction Time by Ethanol on Flavonoid Content and Its Antibacterial Activities. We found that the antibacterial activity of the ethanol extract changed with the extraction time and increased noticeably with the treatment time (Figure 1). There was a positive relationship between the antibacterial activity (the diameter of the inhibition zone using *S. paratyphi* A CMCC 50093) and the total polyphenols (r = 0.88)/total flavonoid content (r = 0.92) of the extract when the fruit was treated for different times with ethanol. However, the relationship between the diameter of the inhibition zone and the tannin content (r = 0.72) was less clear.

Trace Antibacterial Compounds Isolated by TLC and a Bioautographic Method. The flavonoids in the bayberry extract may contribute to the antibacterial activity by determining the inhibitory activity following TLC separation. Four separated spots were obtained by TLC that produced a blue and green color under UV light (254 nm) (Figure 2), and flavonoids commonly show blue or green color under UV light. Among the above four spots, there were three major constituents that produced

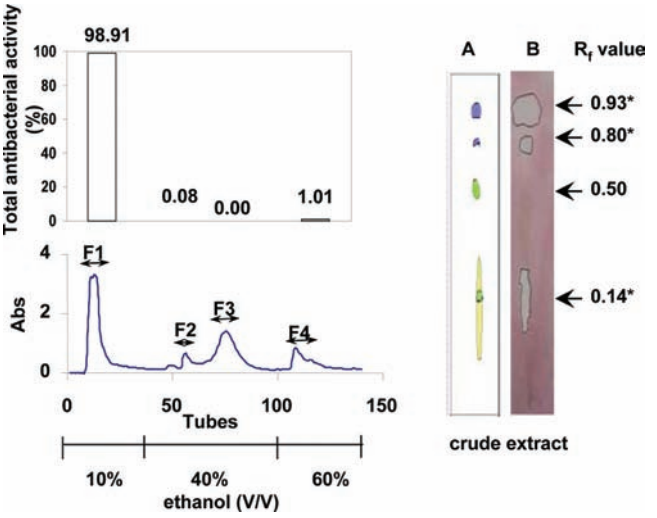

Figure 2. Separation and detection of the fractions with antibacterial activity in the crude extract. TLC plates (right panel) indicating the antibacterial activity of flavonoids. The dots on the plate (A, on the right) showed a blue or green color under 254 nm UV light. The plates (B, on the right) were sprayed with a *S. paratyphi* A CMCC 50093 culture, and the growth was visualized with tetrazolium red. Arrows in the right panel indicate the positions (R_f value) of the major compounds with UV adsorption; *the positions of the major active compounds. The polyamide chromatography profiles (lower panel on the left) and total antibacterial activities against *S. paratyphi* A CMCC 50093 (upper panel on the left). The column (2.5 cm × 30 cm) was pre-equilibrated and eluted using a nonlinear gradient of ethanol solutions as follows: 0−200 min, 10% ethanol (V/V); 200−500 min, 40% ethanol; and 500−650 min, 60% ethanol. Fractions (4.0 mL per tube) were collected at a flow rate of 0.8 mL/min. The eluate was monitored by absorbance at 254 nm.

large inhibitory zones with an R_f of 0.93, 0.80, and 0.14 on the TLC plate when tetrazolium red was incorporated into the media to facilitate the visualization of regions of growth inhibition, and the clear zones against a red-colored background indicated bacterial growth postincubation.[19]

Separation of Flavonoids by a Polyamide Column and the Quantification of the Antibacterial Activity. A total of four fractions with UV absorbance were separated by a polyamide column; among these, three fractions (F1, F2, and F4) showed antibacterial activity with MICs of 0.625, 2.50, and 0.156 mg/mL, respectively. When 8280 mg of BPCE was applied to the column, 5381.3, 16.6, 21.7, and 13.7 mg of fractions F1, F2, F3, and F4 were obtained, respectively. Using the total activity calculation method,[21] the total activities of fractions F1−F4 were analyzed (Figure 2). The highest total activity was observed with the fraction F1 (98.91%). Although the MIC value of the fraction F4 was the highest, the contribution to the total activity was not the greatest because this fraction was the lowest in yield.

Identification of the Fraction F1 by UPLC-ESI-MS. Four phenol compounds were well separated using UPLC and characterized by MS from the fraction F1 with the highest total activity (Figure 3). Peaks A and C were unambiguously identified as cyanidin 3-*O*-glucoside and quercetin-3-*O*-glucoside, respectively, by comparing their retention time, UV−vis spectroscopic data, and the pseudomolecular ion $[M + H]^+$ with the authenticated standards. By comparison with the relevant standards and the data from the literature,[1,4,22] peak B demonstrated a mass typical of myricetin and a fragment ion at m/z 319, and peak D

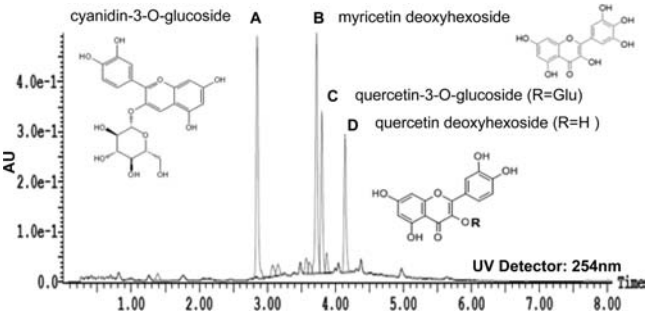

Figure 3. Chromatography of UPLC of the fraction F1 and the structure of the main compounds identified by ESI-MS.

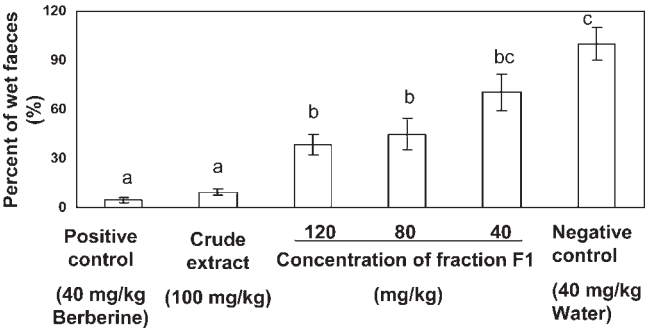

Figure 4. Antidiarrheal effect of bayberry extract on the diarrhea in mice induced by *S. paratyphi* A CMCC 50093. The data are expressed as a percent of the value from the negative control group that received 40 mg/kg water. Bars with the same lower case letter were not significantly different ($p < 0.10$) as determined by ANOVA.

was a typical mass in the positive mode of quercetin aglycone (the MS data shown in the Supporting Information). Thus, peaks B and D were tentatively identified as myricetin deoxyhexoside and quercetin deoxyhexoside, respectively.

Effect of Flavonoids in Mice with Diarrhea Induced by *Salmonella*. The bayberry extract was observed to possess the anti- diarrheal function in the mice with *Salmonella*-induced diarrhea. The BPCE showed a significant effect ($p < 0.10$) at a dose of 100 mg/kg (Figure 4), which was equivalent to 24.2 g of bayberry fruit administrated to one mouse. The weight of one granule is normally ∼20 g; that is, one mouse who injected one more granule of bayberry fruit showed significant antidiarrheal function. The fraction F1 also exerted a significant reduction of the percent of wet feces in the test groups ($p < 0.10$) at the 80 mg/kg dose (Figure 4). The fraction F1 dose used (80 mg/kg) was equal to 89.2 g of bayberry fruit; thus, our research suggests that a dose of approximately 4−5 granules of bayberry fruit would produce a significant reduction of diarrhea in an adult (body weight, 60 kg).

DISCUSSION

In this study, we provide the first report of the main bioactive compounds of the bayberry fruit. We found that flavonoids, including cyanidin-3-*O*-glucoside, myricetin deoxyhexoside, quercetin-3-*O*-glucoside, and quercetin deoxyhexoside, contributed to the antibacterial activity and antidiarrheal function.

We speculate that the antidiarrheal action of the bayberry is correlated with the antibacterial activity, which is consistent with other studies on traditional medicinal plants.[7−9] Moreover, the

antidiarrheal action of quercetin, one of the main compounds in the fraction F1, has been reported, and its mechanism has been illustrated. The mechanisms of the antidiarrheal action of quercetin include inhibition of ileum contractions induced in vitro by transmural electrical stimulation,[23] inhibition of small intestinal peristalsis in vivo, [24] relaxation of intestinal smooth muscle, inhibition of bowel contractions,[25] and/or possession of an antimotility effect that contributes to the antidiarrheal activity.[26] Among the four compounds identified in the fraction F1, three pure components, quercetin, myricetin, and cyanidin, possess antibacterial activity. For example, quercetin shows clear activity against *S. aureus*,[17] and myricetin and cyanidin show activity against *Escherichia coli*.[27] These reports support our results that the fraction F1, which contained these compounds, possesses antidiarrheal function and antibacterial activity simultaneously.

On the basis of the traditional use of bayberry to cure diarrhea and its in vitro antibacterial activity, the mouse model induced by food-borne pathogens was used in our research, which was more realistic than the diarrhea induced by castor oil or magnesium sulfate.[28] So, the bacteria most sensitive to bayberry, *S. paratyphi* A CMCC 50093, was used to induce the diarrhea in mice. Using this model, the in vivo antidiarrheal function of the fraction F1 (with the highest antibacterial activity) showed a significant reduction of diarrhea in a mouse, which would have an equal dose of 4−5 granules of bayberry fruit for an adult human. However, in traditional use, an adult consumes only 1−2 granules of bayberry fruit immersed in distilled spirit, and the symptoms of diarrhea would be significantly improved. This difference in dose may be due to the fact that the sample administered to the mice (the fraction F1) was only one fraction containing the most antibacterial activity of the crude extract and represents only one part of the total granule. In addition, the observation may be due to the loss of another compound during the procedure of extraction and fraction and/or a decrease in the synergistic effects among fractions F1 to F4.

The four main compounds identified in the active fraction of bayberry extract by ethanol are also present in the pomace byproduct of bayberry juice[3] in addition to the bayberry fruit,[4] which belong to the subgroup of flavonoids based on the classification table of the phenolic family in berries,[13] including flavonoids, phenolic acids, lignans, and complex phenolic polymers (polymeric tannins). However, Heinonens[29] has reported that it is the tannin content in the berries that exhibits the antimicrobial properties against pathogenic bacteria, which is different from our results. To ensure the integrity of our experiments, the antibacterial compounds were not only tracked qualitatively by TLC and bioautographic investigation but also separated by a polyamide column and were further quantitatively analyzed using the total activity calculation method during our research.

As compared with the MIC values from the root or stem of the other herbal medicine plants (at the level of μg/mL),[30] the MIC value of the bayberry extract (at the level of mg/mL) was much higher, although it is similar to the MIC values of other berries, such as cloudberry, raspberry, strawberry, and cranberry.[14,17] Similar to these other berries, *Salmonella* was the most sensitive pathogen. *Staphylococcus* was not as sensitive to bayberry as it is to Finnish berries.[14] Our results showed that *L. monocytogenes* showed good sensitivity to the bayberry, which is different from the result of Puupponen-Pimiäs,[14] where the bacterium was only inhibited by cranberry at a higher concentration and was not inhibited by other berries, such as cloudberry, raspberry, or strawberry.

In conclusion, our findings provide scientific evidence for the antidiarrheal function of the bayberry fruit that has been used in traditional medicine for centuries in China. We determined that flavonoids, including cyanidin-3-*O*-glucoside, myricetin deoxyhexoside, quercetin-3-*O*-glucoside, and quercetin deoxyhexoside, contributed to the bioactivity. These results should help to improve the utilization of the bayberry as a pharmacologically acceptable antimicrobial agent or food preservative, although it is only a small step to advancing the understanding of the main active antidiarrheal compounds. Further efforts should be directed at investigating the principle flavonoids found in bayberry.

■ ASSOCIATED CONTENT

Supporting Information. MS data of the main compounds in the fraction F1 with the most antibacterial activity and A−D the four main peaks of the fraction F1 with the highest antibacterial activity. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Fax: +86-510-85329081. E-mail: yaoweirongcn@jiangnan.edu.cn.

Funding Sources

This article is supported by 111 project-B07029 and PCSIRT0627, the Fundamental Research Funds for the Central Universities No. JUSRP21124, 30908, 31005, and 31106, National Key Technology R&D Program in the 11th Five Year Plan of China No. 2009BADB9B04.

■ ACKNOWLEDGMENT

We thank the Wuxi Center for Disease Prevention & Control and the Zhangjiagang Entry-Exit Inspection & Quarantine Bureau for supplying the pathogens.

■ REFERENCES

(1) Fang, Z.; Zhang, M.; Sun, Y. How to improve bayberry (*Myrica rubra* Sieb. et Zucc.) juice color quality: Effect of juice processing on bayberry anthocyanins and polyphenolics. *J. Agric. Food Chem.* **2006**, *54*, 99–106.

(2) Fang, Z.; Zhang, Y.; Yuan, L.; Ma, G.; Chen, J.; Liu, D.; Ye, X. Phenolic compounds and antioxidant capacities of bayberry juices. *Food Chem.* **2009**, *113*, 884–888.

(3) Zhou, S. H.; Fang, Z. X.; Yuan, L.; Chen, J. C.; Liu, D. H.; Ye, X. Q. Phenolics and antioxidant properties of bayberry (*Myrica rubra* Sieb. et Zucc.) pomace. *Food Chem.* **2009**, *112*, 394–399.

(4) Bao, J. S.; Cai, Y. Z.; Sun, M.; Wang, G. Y.; Corke, H. Anthocyanins, flavonols, and free radical scavenging activity of Chinese bayberry (*Myrica rubra*) extracts and their color properties and stability. *J. Agric. Food Chem.* **2005**, *53*, 2327–2332.

(5) Pan, X. R. Study on the antioxidant activity of *Myrica rubra* Sieb. et Zucc. Master Thesis. Zhejiang University: Hangzhou, China, 2002.

(6) Li, S. Z. *Bencao Gangmu (Compendium of Materia Medica)*; The Ming Dynasty of China: China, 1593.

(7) Alanís, A. D.; Calzada, F.; Cervantes, J. A.; Torres, J.; Ceballos, G. M. Antibacterial properties of some plants used in Mexican traditional medicine for the treatment of gastrointestinal disorders. *J. Ethnopharmacol.* **2005**, *100*, 153–157.

(8) Mathabe, M. C.; Nikolova, R. V.; Lall, N.; Nyazema, N. Z. Antibacterial activities of medicinal plants used for the treatment of

diarrhoea in Limpopo Province, South Africa. *J. Ethnopharmacol.* **2006**, *105*, 286–293.

(9) Thakurta, P.; Bhowmik, P.; Mukherjee, S.; Hajra, T. K.; Patra, A.; Bag, P. K. Antibacterial, antisecretory and antihemorrhagic activity of *Azadirachta indica* used to treat cholera and diarrhea in India. *J. Ethnopharmacol.* **2007**, *111*, 607–612.

(10) Lima, Z. P.; dos Santos, R. d. C.; Torres, T. U.; Sannomiya, M.; Rodrigues, C. M.; dos Santos, L. C.; Pellizzon, C. H.; Rocha, L. R. M.; Vilegas, W.; Souza Brito, A. R. M.; Cardoso, C. R. P.; Varanda, E. A.; de Moraes, H. P.; Bauab, T. M.; Carli, C.; Carlos, I. Z.; Hiruma-Lima, C. A. *Byrsonima fagifolia*: An integrative study to validate the gastroprotective, healing, antidiarrheal, antimicrobial and mutagenic action. *J. Ethnopharmacol.* **2008**, *120*, 149–160.

(11) Hu, J. Studies on the antimicrobial activity of leaf extract from *Myrica rubra* Sieb. et. Zucc. Master Thesis, Zhejiang University: Hangzhou, China, 2002.

(12) Fang, Z.; Zhang, M.; Sun, Y.; Sun, J. Polyphenol oxidase from bayberry (*Myrica rubra* Sieb. et Zucc.) and its role in anthocyanin degradation. *Food Chem.* **2007**, *103*, 268–273.

(13) Nohynek, L. J.; Alakomi, H. L.; Kähkönen, M. P.; Heinonen, M.; Helander, I. M.; Oksman-Caldentey, K. M.; Puupponen-Pimiä, R. H. Berry Phenolics: Antimicrobial Properties and Mechanisms of Action Against Severe Human Pathogens. *Nutr. Cancer* **2006**, *54* (1), 18–32.

(14) Puupponen-Pimiä, R.; Nohynek, L.; Hartmann-Schmidlin, S.; Kahkonen, M.; Heinonen, M.; Maatta-Riihinen, K.; Oksman-Caldentey, K. M. Berry phenolics selectively inhibit the growth of intestinal pathogens. *J. Appl. Microbiol.* **2005**, *98*, 991–1000.

(15) Sellappan, S.; Akoh, C. C.; Krewer, G. Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *J. Agric. Food Chem.* **2002**, *50*, 2432–2438.

(16) Pharmacopoeia Commission of the People's Republic of China. *Chineses Pharmacopoeia*; Chemical Industry Press: Beijing, China, 2003.

(17) Rauha, J.-P.; Remes, S.; Heinonen, M.; Hopia, A.; Kähköen, M.; Kujala, T.; Pihlaja, K.; Vuorela, H.; Vuorela, P. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int. J. Food Microbiol.* **2000**, *56*, 3–12.

(18) Randall, L. P.; Cooles, S. W.; Sayers, A. R.; Woodward, M. J. Association between cyclohexane resistance in Salmonella of different serovars and increased resistance to multiple antibiotics, disinfectants and dyes. *J. Med. Microbiol.* **2001**, *50*, 919–924.

(19) Shai, L. J.; McGaw, L. J.; Aderogba, M. A.; Mdee, L. K.; Eloff, J. N. Four pentacyclic triterpenoids with antifungal and antibacterial activity from *Curtisia dentata* (Burm.f) C.A. Sm. leaves. *J. Ethnopharmacol.* **2008**, *119*, 238–244.

(20) Kossah, R.; Zhang, H.; Chen, W. Antimicrobial and antioxidant activities of Chinese sumac (*Rhus typhina* L.) fruit extract. *Food Control* **2011**, *22*, 128–132.

(21) Eloff, J. N.; Jäger, A. K.; Staden, v. J. The stability and relationship between anti-inflammatory activity and antibacterial activity of southern African Combretum species. *S. Afr. J. Sci.* **2001**, *97*, 291–293.

(22) Fang, Z.; Zhang, M.; Wang, L. HPLC-DAD-ESIMS analysis of phenolic compounds in bayberries (*Myrica rubra* Sieb. et Zucc.). *Food Chem.* **2007**, *100*, 845–852.

(23) Capasso, A.; Pinto, A.; Sorrentino, R.; Capasso, F. Inhibitory effects of quercetin and other flavonoids on electrically-induced contractions of guinea pig isolated ileum. *J. Ethnopharmacol.* **1991**, *34*, 279–281.

(24) Megli, R.; Autore, G.; Di Carlo, G.; Capasso, F. Inhibitory action of quercetin on intestinal transit in mice. *Phytother. Res.* **1990**, *4*, 201–205.

(25) Taylor, L. Guava. In *The Healing Power of Rainforest Herbs: A Guide to Understanding and Using Herbal Medicinals*; Taylor, L., Ed.; Square One Publisher: New York, 2005.

(26) Meckes, M.; Torres, J.; Calzada, F.; Rivera, J.; Camorlinga, M.; Lemus, H.; Rodríguez, G. Antibacterial Properties of *Helianthemum glomeratum*, a Plant Used in Maya Traditional Medicine to Treat Diarrhoea. *Phytother. Res.* **1997**, *11*, 128–131.

(27) Puupponen-Pimiä, R.; Nohynek, L.; Meier, C.; Kahkonen, M.; Heinonen, M.; Hopia, A.; Oksman-Caldentey, K. M. Antimicrobial properties of phenolic compounds from berries. *J. Appl. Microbiol.* **2001**, *90*, 494–507.

(28) Uddin, S. J.; Shilpi, J. A.; Alam, S. M. S.; Alamgir, M.; Rahman, M. T.; Sarker, S. D. Antidiarrhoeal activity of the methanol extract of the barks of *Xylocarpus moluccensis* in castor oil- and magnesium sulphate-induced diarrhoea models in mice. *J. Ethnopharmacol.* **2005**, *101*, 139–143.

(29) Heinonen, M. Antioxidant activity and antimicrobial effect of berry phenolics: A Finnish perspective. *Mol. Nutr. Food Res.* **2007**, *51*, 684–691.

(30) Mbosso, E. J. T.; Ngouela, S.; Nguedia, J. C. A.; Beng, V. P.; Rohmer, M.; Tsamo, E. *In vitro* antimicrobial activity of extracts and compounds of some selected medicinal plants from Cameroon. *J. Ethnopharmacol.* **2010**, *128*, 476–481.