

Antioxidant Properties and Anti-inflammatory Effects of the Hydroethanolic Extracts of Two Varieties of Bayberry fruit (*Myrica rubra* Sieb et Zucc.) Prepared by Stirring and Ultrasonic Methods

Kuan-Hung LIN¹, Chun-Ping LU², Jia-Wei CHAO¹, Yi-Ping YU^{3*}

¹Chinese Culture University, Graduate Institute of Biotechnology, Taipei 11114, Taiwan; rlin@faculty.pccu.edu.tw; nsr.tigra@gmail.com

²Fu Jen Catholic University, Department of Food Science, New Taipei City 24205, Taiwan; 089984@mail.fju.edu.tw

³Chinese Culture University, Department of Nutrition and Health Sciences, Taipei 11114, Taiwan; yyp@faculty.pccu.edu.tw (*corresponding author)

Abstract

Chinese bayberry (*Myrica rubra* Sieb. et Zucc.) is an economically important medicinal plant with multiple uses. Two varieties 'Dongkui Oriental Pearl' (Dongkui for short) and *acuminata* 'Nakai' (Nakai for short) were used to compare and evaluate the antioxidant activities of hydroethanolic extracts of the fruit using ultrasonic and stirring extraction methods. Dongkui bayberry fruit extract (BFE) prepared using the ultrasonic method exhibited a significantly higher value for the total phenolic content (TPC) and had lower 50% inhibitory concentration (IC₅₀) values of scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and hydrogen peroxide (H₂O₂), as well as reducing power compared to the other treatment. The TPC of the BFE was significantly correlated with its DPPH, ABTS, and H₂O₂ radical-scavenging and reducing power activities. Dongkui BFE at a concentration of 0.25 mg/mL exhibited significantly greater protection of RAW 264.7 mouse macrophages against H₂O₂-induced damage and lipopolysaccharide (LPS)-stimulated nitric oxide production by macrophages, and it displayed remarkable inhibitory effects compared to the other extracts using the ultrasonic extraction method. Furthermore, compared to the Nakai BFE, macrophages exposed to the Dongkui BFE by the ultrasonic extraction method significantly inhibited LPS-induced production of tumor necrosis factor- α at a concentration of the extract of 0.25 mg/mL. The antioxidant properties and anti-inflammatory and protective effects of BFE prepared by stirring and ultrasonic methods are discussed for the first time in this study.

Keywords: antioxidant activity; bayberry fruit extract; free radical scavenging; *Myrica rubra*; ultrasonics

Introduction

Chinese bayberry (*Myrica rubra* Sieb. et Zucc.), of the Myricaceae family, is native to eastern Asia. Depending on the cultivar, ripe Chinese bayberry fruits can be purple, red, pink, or white, and they all have an attractive flavor and taste sour but delicious. All parts of the bayberry plant are used in Chinese traditional medicine, and the fruits are used to treat vomiting, diarrhea, and abdominal pain (Sun *et al.*, 2012; Silva *et al.*, 2015). Several studies reported that Chinese bayberry fruit is rich in phytochemicals and bioactive compounds that are beneficial to human health due to their antioxidant (Silva *et al.*, 2015), anti-hyperlipidemic (Jurgonski *et al.*, 2008), anti-diarrheal (Yao *et al.*, 2011), anticancer (Yang *et al.*, 2011), anti-

inflammatory (Farrell *et al.*, 2015; Silva *et al.*, 2015), anti-diabetic (Zhang *et al.*, 2016), anti-obesity (Meireles *et al.*, 2016), antibacterial (Ju *et al.*, 2018), and anti-melanogenesis properties (Juang *et al.*, 2018). In addition to the commercial fruit, the consumption of wild and underutilized fruits is also gaining importance owing to their antioxidant contents and consequent health benefits. The aim of studying such underutilized crops is to promote their conservation as potential sources of antioxidants and boost agronomic advancement to economically uplift local farming communities. A number of wild but potentially commercial fruits are available in Taiwan, among which, the red-fruited Chinese bayberry varieties locally known as Dongkui and Nakai are among high-value edible fruits. They are also highly perishable and susceptible to mechanical injury, physiological deterioration, and fungal

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decay (Wang *et al.*, 2010). It would be advantageous to assess the antioxidant properties of these fruits for possible use in creating functional foods or for consideration as potential sources of natural antioxidants.

There is great interest in the use of potent dietary antioxidants in preventive strategies with applications ranging from preventing oxidative reactions in foods and pharmaceuticals to the role of reactive oxygen species (ROS) in chronic degenerative diseases (Farombi *et al.*, 2004). Although several varieties of bayberries are known to be potent sources of antioxidant compounds (Silva *et al.*, 2015), no literature on the antioxidant properties of Chinese bayberry fruit against free radical-induced oxidative stress in *in vitro* cell experiments is presently available. Relationships between antioxidant activities and the prevention of cell oxidative damage by bayberry fruit extracts (BFEs) are not well researched. Extraction is the first step in the commercial isolation of antioxidant components. It is worth exploring essential applications of BFEs in foods which can enhance the nutritional quality and therapeutic value, and also provide income for bayberry producers and the regional economy. In addition, there is a need to find a single or a group of effective extraction methods for bayberry fruit that can produce natural antioxidant agents, which would likely have important applications in the food and medical industries.

Comparisons of extraction processes of bioactive compounds from plants between traditional (maceration, agitation, Soxhlet, and hydrodistillation) and novel (enzyme, microwave, ultrasound-assisted, and using supercritical fluids) were reported (Wang and Weller, 2006; Ghafoor *et al.*, 2011; Radojković *et al.*, 2012; Azmir *et al.*, 2013; Li *et al.*, 2013; Medina-Torres *et al.*, 2017). Vega Arroy *et al.* (2017) showed that ultrasound processes significantly increased the antioxidant capacity and total anthocyanins and phenolic compounds of *x'kijit* (*Renalmia alpinia* Rottb. Maas) peel extracts while reducing extraction times and increasing the energy efficiency compared to Soxhlet extraction. Thus, in this study, we compared and evaluated the antioxidant properties of BFEs using stirring and ultrasonic methods with 85% ethanol, and studied the effects of total phenols, flavonoids, anthocyanins, and antioxidant activities of the two varieties that can protect and inhibit oxidative damage to mouse RAW 264.7 macrophages induced by H₂O₂. In addition, the protective effects of *M. rubra* extracts were also evaluated against lipopolysaccharide (LPS)-stimulated nitric oxide (NO) and the proinflammatory cytokine tumor necrosis factor (TNF)- α .

Materials and Methods

Source of plants and preparation of plant extracts

Two varieties of red bayberry fruits, Nakai and Dongkui, are popular among local residents, and were collected from a commercial orchard in Keelung City, northern Taiwan in July 2015. The cultivars *acuminata* Nakai and Dongkui (*Orient Pearl*) of *Myrica rubra* were identified according to the List of Plants of Formosa ([http://tai2.ntu.edu.tw/PlantInfo/species-name.php?code=302%20001%](http://tai2.ntu.edu.tw/PlantInfo/species-name.php?code=302%20001%2002%200)

2002%200), and voucher specimen number TAIID65696 was deposited at the National Taiwan University herbarium (Yang and Lu, 1996). Dongkui has been planted island-wide and is the most popular variety on the market due to its large fruit size compared to the Nakai variety that originates from Taiwan and is mostly planted in southern Taiwan. All bayberries were randomly harvested according to the shape and uniform color at their commercially mature stage. After being harvested, freshly picked bayberry fruit samples were carefully washed with tap water, lyophilized using a freeze dryer (FD-5060, Panchum Scientific, Taipei, Taiwan), and stored at -20 °C. After milling, 10 g of lyophilized fruit was mixed in separate bottles with 500 mL of ethanol/water (85:15, v/v), followed by treatment with ultrasonic bath shaking (DELTA ultrasonic cleaner DC150H, New Taipei City, Taiwan, at 40 kHz and 150 W) for 6 h at room temperature to ensure that all of the colored ingredients were extracted (Kumoro and Hartati, 2015). For the stirring method, the above-described 85% hydroethanolic extracts were treated by stirring with an electromagnetic stirrer (Corning PC-400D, New York, NY, USA) for 6 h at room temperature. The extracts were then transferred to a rotary evaporator (Buchi R-205, Flawil, Switzerland) under reduced pressure to remove the ethanol until a thick, viscous solution was obtained. Finally, the viscous solutions were frozen at low temperature until solid and then were subjected to a freeze-drying process to remove any water residue. The extraction yield (%) was calculated as the dry extract weight (g) divided by the dry sample weight (g, dry mass) x 100%. The hydroethanolic extract was then stored at -20 °C until being used for the antioxidant activity assays.

Analysis of the total phenol content (TPC), total flavonoid content (TFC), and total anthocyanin content (TAC)

The TPC was assayed from the Nakai and Dongkui BFEs with the Folin-Ciocalteu reagent, using gallic acid as a standard (Lee *et al.*, 2018). Three hundred microliters (μ L) of Folin-Ciocalteu reagent (diluted 10-fold in distilled-deionized water, ddH₂O) and 240 μ L of a 1 M sodium bicarbonate solution were added to 30 μ L of each BFE. After incubation for 15 min at 20 °C, the absorbance at 765 nm was read. The TPC was expressed as mg gallic acid equivalents (GAE)/g dry extract. The linear range of the calibration curve was 27~150 μ g/mL ($r = 0.9988$).

The TFC was determined according to the method described by Kashiwada *et al.* (2005). Six hundred microliters of BFE was mixed with an equal volume of 10% AlCl₃. After incubation for 15 min, the absorbance at 510 nm was read. The TFC, using quercetin as a standard, was expressed as mg quercetin equivalents (QE)/g dry extract. The linear range of the calibration curve was 6.25~50 μ g/mL ($r = 0.9982$).

The TAC was determined according to a previously described procedure by Padmavati *et al.* (1997). Briefly, 100 μ L of the BFE was added to 10 mL of 1% HCl/MeOH and thoroughly mixed. The mixture was placed in the dark at 4 °C for 2 h, after which it was centrifuged at 4 °C and 1000 \times g for 15 min. The absorbance of the supernatant was measured at 530 and 657 nm. TAC (μ mol/g) = $[3 \times (A_{530} - 0.33 \times A_{657})] / 31.6$.

Measurement of the scavenging activities against the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis-(3-ethyl benzothiazoline-6-sulfonic acid) (ABTS) radicals

Scavenging activities against the DPPH radical by fruit hydroethanolic extracts were determined by previously published methods (Lee *et al.*, 2018). Briefly, an aliquot of 70 μ L of serially diluted extracts was added to 140 μ L of 150 μ M DPPH freshly prepared in methanol, mixed well, and left to stand for 30 min at room temperature in the dark to allow the reaction to precede. DPPH radicals are scavenged by antioxidants through the donation of electrons. The color changes from purple to yellow after reduction, which can be quantified by the decrease in absorbance at a wavelength of 517 nm. The scavenging effect (%) = $[1 - (\text{absorbance of a sample}) / (\text{absorbance of a blank DPPH solution})] \times 100$. The blank was treated in the same manner, except that methanol was used instead of a sample, and ascorbic acid was used as the control. The concentration required for a 50% decrease in the absorbance of DPPH radicals (IC_{50}) was calculated as the percent inhibition of DPPH, and each sample was measured with at least three different concentrations in the DPPH test. The IC_{50} was obtained by plotting the percentage of residual DPPH at a steady state as a function of the antioxidant concentration.

Scavenging activities against the ABTS radical cation by fruit hydroethanolic extracts were determined by the method of Re *et al.* (1999). Total antioxidant capacities of hydrophilic antioxidants were determined using the horseradish peroxidase (HRP)-catalyzed oxidation of ABTS. The reaction mixture contained 0.1 mL of 1 mM ABTS, 100 μ L of 44 U/mL peroxidase, 0.1 mL of 0.5 mM H_2O_2 , and 600 μ L of ddH_2O . After 1 h, 0.01 mL of BFE was added to the mixture, and after 10 min, the absorbance was measured at 734 nm. Trolox (TR) was used as a standard. The IC_{50} value was obtained by interpolation from a linear regression analysis.

Measurement of the H_2O_2 -scavenging capacity of the BFE

Hydrogen peroxide was added to each test sample and assayed in a 96-well microplate. Each well contained 100 μ L of 4 mM *p*-nitrophenylboronic acid (NPBA) and 100 μ L of each sample (Lu *et al.*, 2011). Blank solutions were prepared by mixing 100 μ L of a sample solution with an equal volume of 150 mM carbonate/bicarbonate buffer at pH 9.0. The plates were vortexed, and the reactions were then allowed to proceed for 20 min at room temperature. The absorbance at 405 nm was recorded. The absorbance of the blank was subtracted from the absorbance of the respective samples.

Measurement of the reducing power

The reducing power of the extracts was determined according to the method of Lee *et al.* (2018). Briefly, an aliquot of 20 μ L of the hydroethanolic extract was mixed with an equal volume of 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After cooling down on ice, 80 μ L of ddH_2O , 20 μ L of 10% trichloroacetic acid, and 80 μ L of 0.1% ferric chloride were added to the mixture. After 15 min, the absorbance at 700 nm was measured against a blank. The blank was prepared using 95% ethanol with no extract. An increase in the absorbance of the reaction mixture indicated a higher reducing power. Ascorbic acid

was used as a standard. EC_{50} was effective concentration at the absorbance is 0.5.

Protective effect of the BFE against H_2O_2 -induced oxidative stress

RAW 264.7 cells (10^5 cells/well) were incubated overnight in plates containing 50 μ L of the BFE (0.025~2.5 mg/mL), followed by the addition of 50 μ L of 1.2 mM H_2O_2 to each well; this was allowed to react for 24 h at 37 °C. After that, 50 μ L of MTT (0.5 mg/mL) was added and allowed to react for 2 h at 37 °C. After draining, cells were washed with phosphate-buffered saline (PBS), dissolved in 150 μ L DMSO, and monitored for absorbance at 570 nm using a Microplate reader (Molecular Devices SpectraMax 190, San Jose, CA, USA) for cell viability: Cell viability (%) = $[(A_{570} \text{ of the extract-treated group}) / (A_{570} \text{ of the blank group})] \times 100$ (%). The control contained H_2O_2 and no added sample extract. The blank was only DMEM without H_2O_2 or sample extract.

Measurement of the inhibitory effect on NO production

NO, a potentially toxic molecule is released by innate immune cells during pathogenesis, and its production is involved in the response to lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. Therefore, NO liberated from RAW 264.7 macrophages were measured by the Griess assay (Green *et al.*, 1982) in this study. Briefly, RAW 264.7 macrophages (10^5 cells/well) were incubated for 24 h. Cells were then stimulated with LPS (1 μ g/mL), and treated with each individual BFE sample at concentrations ranging from 0.025 to 2.5 mg/mL for 24 h at 37 °C. The medium was then mixed with an equal volume of Griess reagent (Sigma, St. Louis, MO, USA). The amount of NO was quantified by measuring the absorbance at 540 nm using a microplate reader, and was determined using a sodium nitrite standard curve.

Analysis of proinflammatory cytokine secretions as determined using enzyme-linked immunosorbent assays (ELISAs)

Anti-inflammatory effects of the BFE were analyzed in LPS-induced RAW 264.7 cells. Cells were seeded in 96-well culture plates at a density of 10^5 cells/well, followed by incubation for 24 h. Cells were then exposed to 1 μ g/mL of LPS, treated with each individual BFE sample (0.025~2.5 mg/mL), and incubated for 24 h at 37 °C. Levels of TNF- α in the culture media were determined by a Duo Set mouse TNF- α Sandwich ELISA kit, according to the manufacturer's protocols (R&D Systems, Minneapolis, MN, USA). TNF- α standard at 0~2 mg/mL were used to create a standard curve to calculate the TNF- α contents.

Statistical analysis

All analyses were conducted in triplicate, and results are expressed as the mean and standard deviation (SD). An analysis of variance (ANOVA) with Duncan test at 0.05 was performed using the SAS program vers. 9 (SAS Institute, Cary, NC, USA).

Pearson's correlation coefficients of total phenols, flavonoids, anthocyanin, DPPH, ABTS, H_2O_2 , and the reducing power after treatment with BFE were also determined.

Results

Analysis of the TPCs, TFCs, and TACs of the BFEs

The TPCs, TFCs, and TACs of extracts from the two varieties and from the two different extraction methods were analyzed by spectrophotometry (Table 1). The Dongkui variety of BFE contained a significantly higher TPC than that of Nakai. Moreover, the BFE prepared by the ultrasonic method had a significantly higher TPC compared to the stirring method. The highest (18.50 ± 0.29 mg GAE/g extract) and lowest (11.10 ± 0.68 mg GAE/g extract) TPCs were respectively found in the Dongkui sample prepared by ultrasonics and in the Nakai sample prepared by stirring. TFCs of all test samples ranged from 2.23 ± 0.15 (Dongkui prepared by the ultrasonic method) to 2.69 ± 0.44 mg QE/g extract (Dongkui prepared by the stirring method), and no significant differences were detected among the various samples. No significant differences in TACs (which ranged $2.44 \pm 0.42 \sim 2.77 \pm 0.38$ μ mol/g extract) appeared among all test samples regardless of the variety or extraction method. In addition, the extraction yields (%) of the two bayberry varieties using different extraction methods were 40.3% (Nakai by ultrasonics), 38.5% (Nakai by stirring), 41.8% (Dongkui by ultrasonics), and 39.8% (Dongkui by stirring).

Ability of *M. rubra* fruit extracts to scavenge the DPPH, ABTS, and H₂O₂ radicals and their *in vitro* reducing power

Myrica rubra extracts were tested for their antioxidant activities, and results from Table 2 show that IC₅₀ values of DPPH with Dongkui BFEs prepared by both methods ($0.65 \pm 0.01 \sim 0.71 \pm 0.02$ mg/mL) were significantly lower than those of Nakai BFEs ($1.32 \pm 0.11 \sim 1.53 \pm 0.01$ mg/mL). In addition, Dongkui BFEs prepared by both methods also had significantly lower IC₅₀ values of ABTS radical scavenging than the other test samples, and the

Dongkui BFE prepared by sonication had the highest activity compared to the others. The Dongkui BFE prepared by sonication also had the highest H₂O₂-scavenging activity at 1.83 ± 0.13 mg/mL of extract, followed by the Dongkui BFE prepared by stirring (3.97 ± 0.87 mg/mL), and Nakai BFEs prepared by both methods (11.05 ± 0.16 and 11.79 ± 0.68 mg/mL). The BFEs exhibited significant differences in the ferric-reducing antioxidant power as they exhibited a wide range of EC₅₀ values of 2.78 ± 0.09 (Nakai BFE prepared by the stirring method) to 0.39 ± 0.01 mg/mL (Dongkui BFE prepared by the sonication method). Therefore, the Dongkui BFE prepared by sonication displayed the best antioxidant activity in all *in vitro* antioxidant experiments.

Correlation coefficients between *in vitro* antioxidant activities of the BFEs

Correlation coefficients of TFC, TFC, TAC, and the DPPH-, ABTS-, and H₂O₂-scavenging activity assays are shown in Table 3. Significant positive correlations ($r = 0.93 \sim 0.99$) were found among DPPH scavenging, ABTS scavenging, reducing power, and H₂O₂ scavenging. According to the TPC assay, the IC₅₀ of antioxidant activity was significantly and negatively associated with the contents of DPPH, ABTS, and H₂O₂, and the reducing power ($r = -0.80 \sim -0.83$). However, no correlations were observed for the remaining antioxidant activity assays.

Protective effects of BFEs against H₂O₂-induced oxidative stress in RAW 264.7 mouse macrophages

Protective effects of the BFEs against H₂O₂-induced oxidative stress in RAW 264.7 mouse macrophages are illustrated in Fig. 1. All BFEs produced significantly higher cell survivability compared to the control at all extract concentrations except for Dongkui BFE prepared by the ultrasonic method at 0.025 mg/mL.

Table 1. Total phenols (TPC), total flavonoids (TFC), and total anthocyanins (TAC) contents of extracts from two bayberry varieties using ultrasonic and stirring extractions methods

	TPC (mg of GAE/g)	TFC (mg QE/g)	TAC (μ mol/g extract)
Nakai			
Ultrasonics	13.41 ± 1.08^b	2.41 ± 0.26^a	2.44 ± 0.42^a
Stirring	11.10 ± 0.68^c	2.66 ± 0.30^a	2.61 ± 0.12^a
Dongkui			
Ultrasonics	18.50 ± 0.29^a	2.23 ± 0.15^a	2.77 ± 0.38^a
Stirring	14.18 ± 0.28^b	2.69 ± 0.44^a	2.68 ± 0.18^a

Each value is expressed as the mean \pm SD ($n = 3$).

Means with different letters significantly differ ($p < 0.05$) among extraction methods and varieties.

GAE, gallic acid equivalent; QE, quercetin equivalent.

Table 2. Antioxidant activities of extracts of two bayberry varieties using ultrasonic and stirring methods

	DPPH	ABTS	H ₂ O ₂	Reducing power
	IC ₅₀ (mg/mL)			EC ₅₀ (mg/mL)
Nakai				
Ultrasonics	1.32 ± 0.14^b	4.81 ± 0.63^a	11.05 ± 0.16^a	1.70 ± 0.06^b
Stirring	1.53 ± 0.01^a	5.05 ± 0.04^a	11.79 ± 0.68^a	2.78 ± 0.09^a
Dongkui				
Ultrasonics	0.65 ± 0.01^c	1.54 ± 0.01^c	1.83 ± 0.13^c	0.39 ± 0.01^d
Stirring	0.71 ± 0.02^c	2.18 ± 0.01^b	3.97 ± 0.87^b	0.53 ± 0.03^c

Each value is expressed as the mean \pm SD ($n = 3$).

The half-inhibitory concentration (IC₅₀) was calculated as the antioxidant concentration required to provide 50% of the antioxidant activity.

EC₅₀ is the effective concentration used for the reducing power when the absorbance is 0.5.

Means with different letters significantly differ ($p < 0.05$) among extraction methods and varieties.

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Table 3. Correlation coefficients among total phenols, total flavonoids, total anthocyanins, DPPH, ABTS, H₂O₂, and the reducing power of bayberry fruit extracts

	IC ₅₀ of ABTS	EC ₅₀ of the reducing power	IC ₅₀ of H ₂ O ₂	TPC	TFC	TAC
IC ₅₀ of DPPH	0.99**	0.97**	0.97**	-0.80*	0.28 ^{NS}	-0.16 ^{NS}
IC ₅₀ of ABTS	-	0.93**	0.98**	-0.80*	0.27 ^{NS}	-0.20 ^{NS}
EC ₅₀ of Reduction power	-	-	0.93**	-0.82*	0.28 ^{NS}	-0.19 ^{NS}
IC ₅₀ of H ₂ O ₂	-	-	-	-0.83*	0.31 ^{NS}	-0.27 ^{NS}
TPC	-	-	-	-	-0.35 ^{NS}	0.34 ^{NS}
TFC	-	-	-	-	-	0.47 ^{NS}

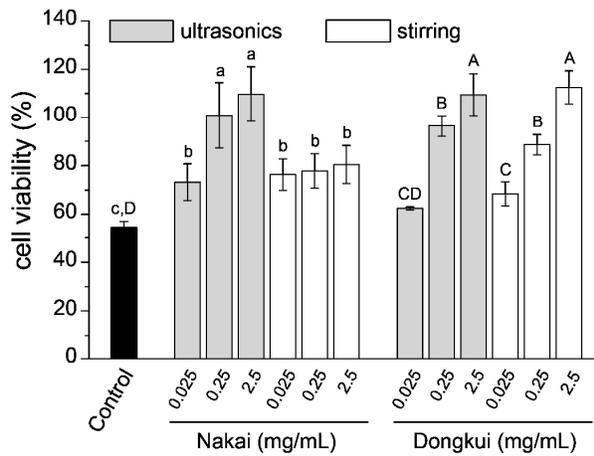
* and ** indicate $p < 0.05$ and < 0.01 , respectively, for each correlation coefficient.TPC, total phenolic content; TFC, total flavonoid content; TAC, total anthocyanin content; IC₅₀, 50% inhibitory concentration; EC₅₀, effective concentration used for the reducing power when the absorbance in 0.5; NS, non-significant difference.

Fig. 1. Protective effects of extracts of two bayberry varieties using different extraction methods against hydrogen peroxide-induced oxidative stress in RAW 264.7 cells. Each value is expressed as the mean \pm SD ($n = 3$). Among six treatments for each extraction method of the same variety compared to the control, means with the same lowercase and capital letter in the 'Nakai' and 'Dongkui' BFEs, respectively, do not significantly differ by Duncan test at $p \leq 0.05$. Cell viability (%) = $[(A_{570} \text{ of extract-treated group}) / (A_{570} \text{ of blank group})] \times 100$. The blank was DMEM only without H₂O₂ or sample extracts. The control was H₂O₂ only without sample extracts

Among them, the Nakai BFE prepared by the ultrasonic method at both 0.25 and 2.5 mg/mL showed the best protective effects against H₂O₂-induced oxidative stress.

In addition, significantly higher cell viability values with the Dongkui BFE prepared by both methods were observed at the concentrations of 0.25 and 2.50 mg/mL compared to the control.

Inhibitory effects on NO contents

In order to examine the anti-inflammatory potential, the inhibitory effects of various BFEs on the production of NO were evaluated in LPS-stimulated RAW 264.7 macrophages. Nitrite, a stable oxidized form of NO produced by inducible NO synthase (iNOS), was measured

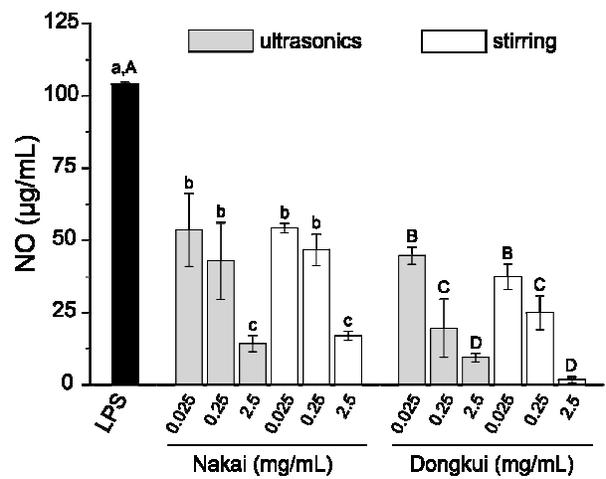


Fig. 2. Nitric oxide (NO) contents of extracts of two bayberry varieties using different extraction methods against lipopolysaccharide (LPS)-induced inflammation of RAW 264.7 cells. Each value is expressed as the mean \pm SD ($n = 3$). The control was only LPS without sample extract. Among six treatments for each extraction method of the same variety compared to the control, means with the same lowercase and capital letter in the 'Nakai' and 'Dongkui' BFEs, respectively, do not significantly differ by Duncan test at $p \leq 0.05$.

as a proxy for NO production in the medium. As shown in Fig. 2, all BFEs produced significantly higher inhibitory effects compared to the control.

At a concentration of 2.5 mg/mL, the Nakai NBE prepared by both the ultrasonic and stirring methods produced relatively lower amounts of NO at 14.18 ± 2.84 and 16.88 ± 1.67 $\mu\text{g/mL}$, respectively. At a concentration of 2.5 mg/mL, the Dongkui BFEs prepared by both the stirring and ultrasonic methods displayed remarkable inhibitory effects at 1.68 ± 1.07 and 9.38 ± 1.47 $\mu\text{g/mL}$, respectively. In addition, the inhibitory effect gradually increased as the Dongkui BFE concentration increased, suggesting that the BFE inhibited NO production in a dose-dependent manner.

Effects of BFEs on TNF- α production

Fig. 3 shows the effect of treatments with various BFEs on TNF- α production measured by an ELISA in supernatants of RAW 264.7 cells treated with extracts for 24 h before LPS stimulation. There were no significant differences between the control and Nakai BFEs prepared by either extraction method at any concentration, except for the Nakai BFE prepared by the ultrasonic method at 2.5 mg/mL, which showed an inhibitory effect on the TNF- α content. As the BFE concentration increased, macrophages treated with the Dongkui BFEs prepared by both methods induced significantly higher inhibitory effects on TNF- α , indicative of a dose-dependent response. Compared to the stirring method, the Dongkui BFE prepared by the ultrasonic method at concentrations of 0.25 and 2.5 mg/mL produced significantly higher inhibitory effects on TNF- α secretion against LPS-induced inflammation in RAW 264.7 cells. Furthermore, compared to the Nakai BFE, macrophages treated with the Dongkui BFE induced relatively lower levels of TNF- α at extract concentrations of 0.25 and 2.5 mg/mL.

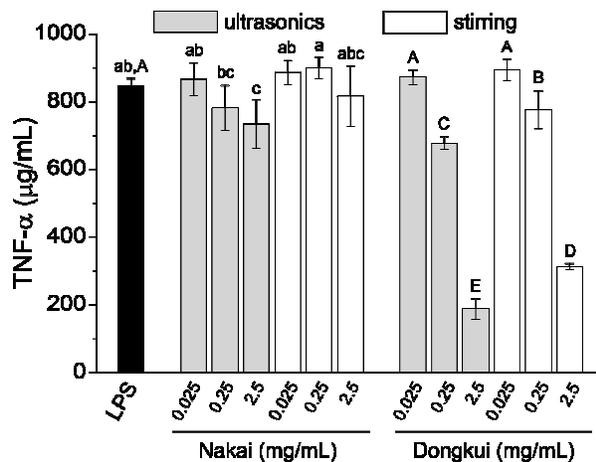


Fig. 3. Tumor necrosis factor (TNF)- α contents of extracts of two bayberry varieties using different extraction methods against lipopolysaccharide (LPS)-induced inflammation of RAW 264.7 cells. Each value is expressed as the mean \pm SD ($n = 3$). The control was only LPS without sample extract. Among six treatments for each extraction method of the same variety compared to the control, means with the same lowercase and capital letter in the 'Nakai' and 'Dongkui' BFEs, respectively, do not significantly differ by Duncan test at $p \leq 0.05$.

Discussion

Different bayberry fruit varieties subjected to different extraction methods showed antioxidant activities, proving their capacity to scavenge DPPH, ABTS, and H₂O₂ radicals and their reducing power. Apparently, the Dongkui BFE had higher antioxidant contents compared to the Nakai BFE, suggesting that different BFE samples can play important roles in antioxidant activity. Antioxidant abilities of the Dongkui BFE prepared by both extraction methods were significantly higher than those of the Nakai BFE. In

particular, the Dongkui BFE prepared by sonication had higher scavenging efficacies for DPPH, ABTS, and H₂O₂ radicals compared to the other test samples, reflecting the relevance of evaluating the major mechanisms of antioxidant actions in cell protection. Moreover, the Dongkui BFE prepared by sonication is an electron donor and can react with free radicals, convert them to more-stable products, and terminate radical chain reactions. The known mechanisms of antioxidants are to provide hydrogen or to act as chelating agents to react with free radicals and then form stable molecules to stop free radical reactions. The antioxidant activity of the BFE involves scavenging free radical ions, thereby inhibiting their oxidation ability.

In our study, the reducing power activities and IC₅₀ values of DPPH, ABTS, and H₂O₂ radicals and the EC₅₀ of the reducing power by the BFEs were highly and significantly associated with the TPC ($r = -0.80 \sim -0.83$), indicating positive relationships among DPPH \bullet -, ABTS \bullet -, and H₂O₂ radical-scavenging activities, reducing power values, and the TPC. These results suggest that phenolic compounds are effective hydrogen donors. Therefore, the excellent antioxidant activities of the BFEs indicate high TPCs, and the test samples have great potential for development into phenolic-rich functional food products. Our results are consistent with previous studies (Zhou *et al.*, 2009; Huang *et al.*, 2014), and suggest that there is a strong correlation between antioxidant activities and TPCs of Chinese bayberry. In addition, Chen *et al.* (2016) reported that various amounts of phenolics (0.47~2.31 g GAE/kg) and flavonoids (0.34~2.08 g rutin equivalents/kg) were found in all tested bayberry varieties. The antioxidant activity of bayberry was highly correlated with the phenolic contents, including flavonoids. Hong *et al.* (2008) demonstrated that the ferric-reducing ability exhibited good linear correlations with the antioxidant activity and TFCs or TPCs in *Eriobotrya* species. Using other antioxidant evaluation methods based on different mechanisms to evaluate the antioxidant activity may produce different results even from the same sample. Furthermore, different extraction solvents resulted in differences in the compositions of the extracts, and consequently their antioxidant activities (Pinelo *et al.*, 2004). In general, antioxidant activities of functional compounds are highly associated with the analytical methods, and it is difficult to directly compare and interpret patterns of antioxidant activities from various studies. In our study, the polarities of the BFEs differed, which could have influenced their uptake rates and cellular distributions. Further work related to characterizing the complete chemical composition of the samples by liquid chromatography-mass spectrometry needs to be conducted.

Purification, separation, and isolation of bioactive compounds from plants are techniques that have undergone new developments in recent years. The goal when searching for bioactive compounds is to find an appropriate method to screen the source material for its bioactivity, such as antioxidant or cytotoxic properties, combined with simplicity, specificity, and speed. However, little information has been reported on the effects of solvents and the ultrasonic extraction method for Chinese bayberry bioactive compounds. Ultrasound is used to disrupt plant cell walls, which helps improve the solvent's ability to

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penetrate cells and obtain higher extraction yields. A smashed sample is mixed with a suitable solvent and placed into an ultrasonic bath, while controlling the temperature and extraction time, and a high extract quality of compounds can be maintained (Garcia-Salas *et al.*, 2010). Corrales *et al.* (2008) showed that ultrasound can break down grape tissues, and it worked well during the production process and released active compounds in solvents with high efficiency. Mulinacci *et al.* (2004) confirmed that an ultrasonic method was most effective for extracting phenolic compounds from strawberries compared to other extraction methods. In our study, total phenols from the Dongkui BFE prepared using an ultrasonic method were shown to possess potent *in vitro* antioxidant activity, which should stimulate research into the contents, capacities, and functions of antioxidant systems in *M. rubra* plants.

H₂O₂ can be used as a mediator to induce cytotoxicity, which will create intracellular redox interference, thereby causing perturbations in the antioxidant defense system. The consistent *in vitro* antioxidant capacity of bayberry cultivars further stimulated us to investigate the ROS-scavenging capacity of H₂O₂-induced cellular ROS in RAW 264.7 cells. Thus, a concentration of as low as 0.025 mg/mL can be used in further studies. Furthermore, a higher value for the cell viability indicates the higher H₂O₂-scavenging activity of the BFE. The RAW 264.7 murine macrophage cell line was used in this study because it has been widely applied to antioxidant research of traditional herbs (Tuntipopipat *et al.*, 2009). Macrophages are considered to play key roles in local host defense. Inflammatory mediators, such as NO and TNF- α , are essential for the host defense system, but excessive production of these mediators at an inflammatory site may cause chronic diseases (Thun *et al.*, 2004). Thus, inhibition of activation of these cells appears to be an important target for treating inflammatory diseases (Lin and Karin, 2007). LPS can stimulate RAW 264.7 macrophages to mimic the condition of excessive ROS production. During LPS stimulation, NO is produced by iNOS (Kubes and McCafferty, 2000), and inhibition of iNOS expression and its activities is well known to be a target for preventing inflammatory diseases and cancer (Surh and Na, 2008). Oxidative damage to DNA is one of the most important mechanisms for initiating carcinogenesis, mutagenesis, and cytotoxicity (Prieto *et al.*, 1999). Such damage is usually caused by hydroxyl radicals, the most reactive compound among ROS. The Fenton reaction is complex and capable of generating both hydroxyl radicals and higher oxidation states of ferric iron (Winterbourn, 1995). Yen *et al.* (2006) revealed the free radical-scavenging and protective effects of lotus seed extracts against reactive nitrogen, sodium nitroprusside, peroxynitrite-induced cytotoxicity, and DNA damage in the macrophage RAW 264.7 cell line. Chao *et al.* (2015) also reported that the ethanolic extract of red bean (RBE) can diminish H₂O₂-induced oxidative damage in RAW 264.7 macrophages. Phenolic compounds and cyanidin-3-O-glucoside from the RBE may have efficacy as overall *in vitro* anti-inflammatory and antioxidant agents. Hwang *et al.* (2016) investigated the antioxidant activities of *Cornus officinalis* in RAW 264.7 cells, and showed that protein

expressions of antioxidant enzymes (Cu/Zn superoxide dismutase (SOD), MnSOD, catalase, and glutathione peroxidase) increased upon treatment with *C. officinalis* extracts. They suggested the therapeutic potential of the *C. officinalis* extract as an antioxidant agent. In the present study, the Dongkui BFE prepared by stirring and ultrasonic methods produced the least amount of NO secretion, and therefore had the best capacity against H₂O₂. The Dongkui BFE offered protection against H₂O₂-induced damage in RAW 264.7 cells without affecting the cell viability, and this protective effect is considered, in large part, to be related to the various antioxidants it contains. Total phenolic compounds in the Dongkui BFE may be a major contributor to inhibition of the formation of ROS, and showed anticancer potential in RAW 264.7 cells. Total phenolic compounds are widely recognized as natural molecules with anti-inflammatory effects. Positive correlations were found between the TPC and some anti-inflammatory effects of different mushroom extracts (Kim *et al.*, 2008). Therefore, the Dongkui BFE can be used to prevent and treat oxidative stress-related disorders. Further studies should focus on the valuable outcomes of its effects in animal models.

The total flavonoid intake of an average American is approximately 20 mg/day, and for Japanese it is around 63 mg/day from plant-based foods (Beecher, 2003). Therefore, the antioxidant effect of the BFE by various extraction methods can be valuable, particularly for finding an inexpensive source of natural antioxidants and functional foods. The Dongkui BFE prepared using ultrasonics revealed the potential to be developed as an active ingredient or food additive, thereby increasing the economic value of Taiwanese bayberries for the food industry. *Myrica rubra* plants are abundant on the island, and the fruit that is marketed in Taiwan varies widely in antioxidant potentials and thus may impart different health benefits to consumers. Bayberry fruits with high antioxidant values prepared by ultrasonic extraction can be used to develop products with high nutraceutical value, which can play significant roles in providing good nutrition and improving human health. People can consume the BFE as a low-cost, nutritious food and use it as a low-cost medicine to treat various diseases. The obvious antioxidant activity of the Dongkui BFE prepared using ultrasonics confirms its important role in the bioactivity of *M. rubra* plants. Determination of the TPC of the BFE is valuable in increasing the bioavailability of bayberry products, and the effective dose in *in vivo* studies is worthy of further investigation.

Conclusions

We identified for the first time that Taiwanese bayberry fruit contains potent polar antioxidants that are responsible for protecting against oxidative damage. *Myrica rubra* variety Dongkui showed higher TPCs and antioxidant activities compared to Nakai. Different extraction methods of the BFEs displayed variable levels of antioxidant substances, and the Dongkui BFE prepared using ultrasonics possessed higher amounts of total phenols and exhibited high free radical-scavenging activities as evaluated using DPPH, ABTS, H₂O₂, and the reducing power. In

addition, the 85% ethanolic BFE was proven to be non-cytotoxic and to have significant anti-inflammatory effects in a mouse RAW 264.7 cell model. These strong oxidation-preventive and radical-scavenging activities of the BFE may be associated with its rich content of total phenols. BFEs exhibited higher *in vitro* and *ex vivo* antioxidant activities and possessed protective capabilities for the biological membrane system. Therefore, the BFE can be used to prevent and treat oxidative stress-related disorders. Further studies are warranted to isolate and identify individual phenolic compounds from the alcoholic extract of *M. rubra* fruits to better understand the biological mechanisms. Overall, we believe that the hydroalcoholic extract of *M. rubra* fruit can be a multifunctional food additive and can be widely applied in the field of medicine in the near future.

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