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ARTICLE

Structural Identification and Bioactivities of Red-Violet Pigments Present in *Basella alba* Fruits

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Mature *Basella alba* L. fruit, with dark blue skin and deep red-violet flesh, is a potential source of natural colorants. Its pigment components and bioactivities deserve particular attention and investigation. In this study, fruit flesh was extracted with 80% methanol (containing 0.2% formic acid) and subjected to solid-phase extraction, semipreparative HPLC isolation, mass spectrophotometric analysis, and structural elucidation. The major red pigment was identified as gomphrenin I. Its quantity increased with the increase of fruit maturity. The gomphrenin I extract yield from ripe fruits was 36.1 mg/100 g of fresh weight. In addition to gomphrenin I, betanidin-dihexose and isobetanidin-dihexose were also detected. The antioxidant activities of gomphrenin I determined by Trolox equivalent antioxidant capacity (TEAC), α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, and antioxidative capacity assays were equivalent to 534 μM Trolox, 103 μM butylated hydroxytoluene (BHT), 129 μM ascorbic acid, and 68 μM BHT at 180, 23, 45, and 181 μM, respectively. The anti-inflammatory function was tested at concentrations of 25, 50, and 100 μM in murine macrophages stimulated with lipopolysaccharide (LPS). The results revealed that gomphrenin I suppressed LPS-induced nitric oxide (NO) production in a dose-dependent manner and decreased PGE_2 and IL-1β secretions at the highest concentration tested. The transcriptional inhibitory activities of gomphrenin I on the expression of inflammatory genes encoding iNOS, COX-2, IL-1β, TNF-α, and IL-6 were also observed. It is of merit to identify gomphrenin I as a principal pigment of *B. alba* fruits and as a potent antioxidant and inflammatory inhibitor. These findings suggest that *B. alba* fruit is a rich source of betalains and has value-added potential for use in the development of food colorants and nutraceuticals.

KEYWORDS: *Basella alba* L.; betanin; gomphrenin I; antioxidant; anti-inflammation

INTRODUCTION

Natural pigments from plants have attracted great attention for their usefulness, not only in the food and cosmetic industries but also in nutraceutical and pharmaceutical developments (*1*−*3*). Betalains are one of the most important pigments, providing a wide range of colors in leaves, fruits, and roots, as well as being involved in plant adaption against exogenous stress (*4*−*6*). In contrast to other pigments, such as anthocyanins, carotenoids, and chlorophylls, betalains have not been extensively studied. This is largely due to the restricted occurrence of betalains in nature. Betalains and anthocyanins, the two most abundant water-soluble pigments, are mutually exclusive and have never been found simultaneously in the same plant (*7*). However, various sources of betalains have been studied, including beetroot (*Beta vulgaris*), *Gomphrena globosa* flowers, *Basella rubra* fruits, Amaranth plants, common cockscomb (*Celosia argentea*), Swiss chard (*B. vulgaris* L.), *Opuntia ficus-indica* fruits, *Hylocereus polyrhizus* fruits, and *Mammillaria* fruits (*8*−*17*).

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Betalains are water-soluble nitrogen-containing natural pigments, which comprise red-violet betacyanins and yellow betaxanthins (*2*, *3*, *6*). Betanins, the major betacyanins in red beetroot, are commonly used colorants in the food industry (*1*, *3*, *5*, *6*, *18*, *19*). The higher stability at pH range 3−7 makes betalains better coloring agents than anthocyanins in acidic and neutral food products. In addition to coloring characteristics, diverse biological activities of betalains have also been investigated, among which antioxidant and chemoprevention activities have been tested most extensively and are well recognized (*1*, *3*−*7*, *20*, *21*). It has been shown that some members of the betalain pigments have higher antioxidative activity compared to typical natural antioxidants such as ascorbic acid, rutin, and catechin (*21*). Beetroot has been used in different folk remedies for immune modulation and for the treatment of cancers and liver and renal diseases (*22*). Intake of beetroot juice has been shown to alleviate the inflammatory process in human peripheral blood mononuclear cells (PBMCs) (*23*). Health-promoting properties of betalain-rich extracts from various sources include radioprotection (*24*), low-density lipoprotein protection (*25*), cancer chemoprevention (*22*, *26*−*29*), and anti-inflammation (*30*, *31*).

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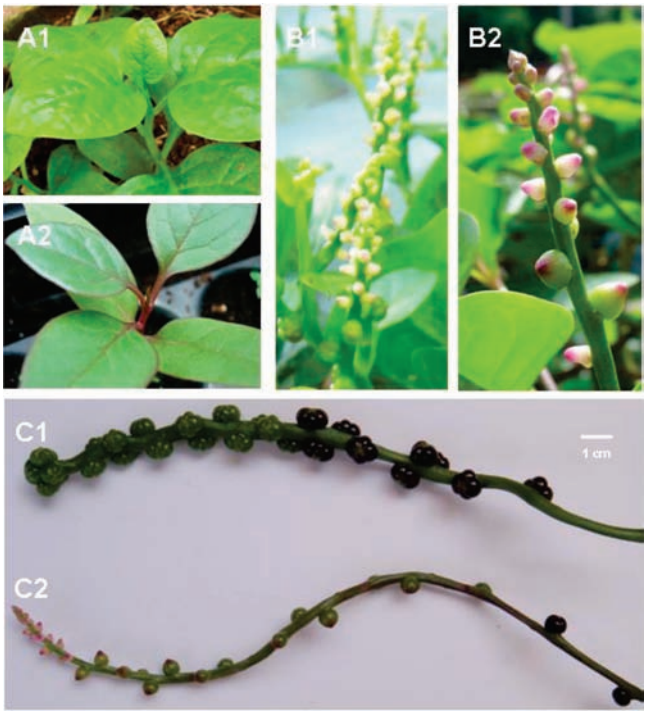

Figure 1. Photographs of *Basella alba* (1) and *Basella rubra* (2): (**A1**, **A2**) young plants; (**B1**, **B2**) flowers and immature fruits; (**C1**, **C2**) bunches containing fruits of various maturities.

Therefore, it is worthwhile to explore and identify new sources of betalains.

Basella alba L. (**Figure 1**), bearing deep-green leaves, is commonly grown and harvested as a novel leafy vegetable in Taiwan, particularly during the summer season when the production of other leafy vegetables is short. Grown into the fall and winter seasons, the vines produce a substantial quantity of fleshy and dark blue fruits, which are usually discarded by farmers. The estimated production of fresh fruits is approximately 2 kg per plant. Value-added use of the natural pigments from the fruits as colorants and/or bioactive ingredient in related product development deserves research interest. The pigment components in *B. rubra* fruits, a red-leaf variety of the *Basella* genus (**Figure 1A2**, **B2**, **C2**), have been identified. Gomphrenin I (15*S*-betanidin 6-*O*-β-glucoside) is a major betacyanin pigment found in *B. rubra* fruits and copresent with minor gomphrenin II [15*S*-betanidin 6-*O*-[6′-*O*-(4-coumaroyl)]-β-glucoside], isogomphrenin I (15*S*-betanidin 6-*O*-β-glucoside), and gomphrenin III (15*S*-betanidin-6-*O*-[6′-*O*-feruloyl-β-glucoside]) (*8*, *9*). In comparison, there is only limited information available about *B. alba* pigments, and in some documents *B. alba* is even mistaken as *B. rubra*. Therefore, component verification, structure identification, and biological activity characterizations of the pigments present in *B. alba* fruits deserve further investigations. In this study, the red-violet pigments of *B. alba* fruits as affected by maturity were determined. Mature fruits were collected and subjected to pigment extraction, isolation, and identification. The assessments of antioxidant activity and anti-inflammatory function of the major pigment were also conducted.

MATERIALS AND METHODS

Plant Material. *B. alba* L. plants were grown at a farm located in Chiayi, Taiwan. Mature (ripe) fruits were harvested and frozen immediately at −20 °C until pigment extraction and chemical analyses were performed. For preparing reference compounds for pigment identification, mature fruits of a native *B. rubra* variety grown in the mountain area of Tungshiao, Miaoli, Taiwan, were harvested and processed following the same procedure as that for the fruits of *B. alba* variety.

***Basella alba* Fruit Red Pigment Analysis at Various Maturities.** *B. alba* fruits (**Figure 1**) at different maturity stages were collected. The fruits were classified into three sublots on the basis of their location and skin color. Whole green color, partial purple color, and whole dark blue color fruits were categorized into immature, partially mature, and ripe sublots, respectively. Each of fruits was manually squeezed to remove one small seed. The seed to fresh fruit weight ratio and flesh moisture content were 15.3 ± 1.4 and $95.9 \pm 0.2\%$, respectively. Fresh flesh was weighed and homogenized in deionized water (1:5, w/v) using a polytron (PT3000, Kinematica AG), at 12000 rpm for 4 min, followed by centrifugation. The supernatant (extract) was filtered through a 0.45 μm membrane prior to high-performance liquid chromatography (HPLC) analysis.

HPLC analyses were conducted using an HPLC pump equipped with a C18 column (Hypersil ODS, 250 × 4.6 mm, 5 μm, Thermal Hypersil Ltd., Cheshire, U.K.) and a UV detector or a photodiode array detector (L-7100 pump, L-7420 UV detector, and L-7455, a photodiode array detector) (Hitachi Co., Ltd., Tokyo, Japan). Twenty microliters of extract was injected and run using a two-solvent system containing (A) 0.1% trifluoroacetic acid in water and (B) 100% methanol. The gradient solvent program was set at 0 min, 85% A and 15% B; 10 min, 70% A and 30% B; 15 min, 20% A and 80% B; 17 min, 10% A and 90% B; 25 min, 9% A and 91% B; 26 min, 0% A and 100% B; and 28 min, 85% A and 15% B, with a flow rate of 1.0 mL/min. The pigment components were detected by absorbance at 280 or 530 nm. An authentic betanin (purified from red beets and diluted with dextrin) purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) was used as a reference standard.

Pigment Isolation by Semipreparative HPLC. For scaled-up violet-red pigment extraction, 50 g of mature fruit flesh, as a batch, was homogenized in 250 mL of 80% aqueous methanol solution (containing 0.2% formic acid) (1:5, w/v), using a polytron (PT3000, Kinematica AG) at 12000 rpm for 4 min. In process optimization, due to the concentration and purification, the extraction medium was changed from water to methanol solution. The resulting suspension was filtered through a Whatman no. 1 filter paper. The filtrate was dried at 30 °C in a rotary vacuum evaporator. The dried residue was dissolved in 10 mL of deionized water and filtered through a 0.45 μm membrane and a C18 solid phase extraction (SPE) cartridge (Supelco Inc., Bellefonte, PA) for pretreatment. The obtained pigment solution was subjected to semipreparative HPLC fractionation using an HPLC system consisting of a pump (model 501, Analytical Scientific Instruments, El Sobrante, CA) and an L-2420 UV detector (Hitachi Co., Ltd., Tokyo, Japan) equipped with a C18 column (Hyperprep HS C18, 250 × 10 mm, 8 μm, Thermo Hypersil Ltd., Cheshire, U.K.). An isocratic solvent system of 20% methanol containing 0.2% formic acid was applied at a flow rate of 2.5 mL/min. The injection volume of the pigment solution was 0.25 mL. The absorbance was monitored at 530 nm.

As resolved by semipreparative HPLC, three peaks were detected and the substance under each of the peaks was collected, pooled, lyophilized, and weighed for determination of yield and further chemical structure identification. The major pigment of peak 3 was subjected to thermal stability determination and bioactivity characterization. The purity of the pigments was checked by high-performance liquid chromatography-diode array detector (HPLC-DAD) analyses after they had been dissolved in an appropriate amount of deionized water. As preliminarily observed, the purity of each pigment as expressed by the percentage of individual peak area to the sum of all detected peaks area at 280 nm was >98%.

ESI-MS Analyses of the Isolated Pigments. MS analyses were conducted by a mass spectrometer (Waters Micromass ZQ 4000 LC mass spectrometer, Waters Co., Milford, MA) run with an electron spray ionization (ESI) type and positive ion polarity. The source temperature, gas flow of nitrogen, capillary voltage, and scan range were 100 °C, 250 L/h for dessolvation and 200 L/h for cone, 3.9 kV, and m/z 200−800, respectively. The resulting ion fragments were compared with those of referenced compounds of betacyanins reported in the literature (*3*, *8*, *10*, *13*, *15*). The major pigment isolated from *B. alba* fruits was identified as gomphrenin I. The structures were elucidated mainly on the basis of a comparison of the HPLC retention times and a match of the molecular weight and ion fragments obtained from mass spectrometry with those of betacyanins isolated from *B. rubra* fruits and red sugar beets (*8*−*10*, *13*).

Thermal Stability and Antioxidant Activity Determination. Freeze-dried powder of peak 3 was dissolved in deionized water to a concentration of 25 μg/mL for thermal stability determination. Aliquots (2.5 mL) of the solution were transferred into screw-cap test tubes and subjected to thermal stability testing at different temperatures, namely, 4 °C (refrigerator), 20 °C (growth chamber), 60 °C (heating module, Resti-Therm, Pierce Chemical Co., Rockford, IL), 90 °C (heating module), or 120 °C (heating module) for 0, 1, 10, 30, 60, and 120 min. At each time point, the tubes were quickly cooled in an ice bath, followed by tempering at ambient temperature and absorbance determination at 530 nm. Relative stability was determined by dividing the absorbance after thermal treatment by the absorbance before the treatment and multiplying by 100.

$ABTS^{\bullet+}$ scavenging activity was assessed according to the procedure of Li et al. (*32*) with modification. Briefly, $ABTS^{\bullet+}$ radical cation was generated by a reaction of 14 mM ABTS with 4.9 mM potassium persulfate. The reaction mixture was allowed to stand in the dark for 16 h at room temperature and used within 2 days. The $ABTS^{\bullet+}$ solution was diluted with ethanol to give an absorbance of 0.700 ± 0.050 at 734 nm. All samples were diluted appropriately to give absorbance values 20−80% of that of blank, from which aliquots (15 μL) of the diluted samples were mixed with 1.9 mL of diluted $ABTS^{\bullet+}$ solution. The mixture was allowed to stand for 6 min at room temperature, and the absorbance at 734 nm was immediately determined. Trolox solution at concentrations ranging from 0 to 15 μM was used to construct a reference curve and equivalency estimation expressed as micromolar Trolox.

For determination of α,α-diphenyl-β-picrylhydrazyl (DPPH) scavenging activity, the reported procedure (*33*) was followed with a minor modification. Lyophilized residue was rehydrated with methanol for each measurement. The diluted solution (2 mL) was mixed thoroughly, in the dark, with 0.1 mL of DPPH solution (2 mM in methanol) at 26 °C for 30 min. The absorbance was measured at 517 nm. Butylated hydroxytoluene (BHT) solutions with different concentrations were prepared in methanol and served as references. To subtract background absorbance from the red pigment molecules, each of the pigment solutions was mixed with 0.1 mL of methanol and incubated concurrently to detect its background absorbance at 517 nm.

Antioxidative potency (AOP) determination was performed as described previously (*34*). The lyophilized extract was dissolved in 1.0 mL of methanol and subjected to determination. The BHT solutions were prepared as described above and served as references.

The procedure of Yen and Chen (*35*) was followed with modification to determine the reducing power. Briefly, lyophilized residue was rehydrated with deionized water. The diluted solution (0.5 mL) was mixed thoroughly with 0.5 mL of 0.2 M phosphate buffer (pH 6.6) and 0.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then, 0.5 mL of 1% trichloroacetic acid was added, and the mixture was centrifuged (1000*g* at 20 °C, 10 min). The upper layer of the solution was mixed with deionized water and 0.1% $FeCl_3$ at a ratio of 1:1:2. The absorbance was measured at 700 nm. An increase in the reaction mixture absorbance indicates increased reducing power. A series of ascorbic acid solutions at different concentrations served as references.

Cell Line and Cell Culture. The murine macrophage cell line, RAW 264.7, was obtained from the Bioresource Collection and Research Center (BCRC, FIRDI, Hsinchu, Taiwan). Cells were routinely maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37 °C under 5% CO_2 in a humidified atmosphere.

To evaluate the gomphrenin I anti-inflammatory activity, RAW 264.7 cells were seeded onto 100 mm culture plates and grown for 24 h in medium containing different concentrations (0, 25, 50, and 100 μM) of gomphrenin I with or without lipopolysaccharide (LPS) under the incubation conditions described above. At the end of treatment, the medium was collected for inflammatory mediator measurements, and the cells were harvested for gene expression analyses.

Cell Viability Assay. For cytotoxicity evaluation, cells were seeded in 0.2 mL of medium at a density of 5×10^5 cells/mL into a 96-well microplate and cultured for 24 h in the medium containing different concentrations (0, 25, 50, and 100 μM) of gomphrenin I with or without 0.5 μg/mL of LPS. Cell viability was determined using the 3-(4,5-dimethylthiahiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (*36*, *37*). Four hours before the end of the treatment, 10 μL of MTT solution (5 mg/mL) was added to each well. The incubation was continued at 37 °C. The intracellular dark-purple formazan derived from the tetrazolium salt was then dissolved in 100 μL of dimethyl sulfoxide (DMSO). The absorbance of the cell solution at 570 nm was measured in a microplate spectrophotometer. The absorbance was proportional to the number of viable cells.

Nitric Oxide (NO) Measurement. Nitrite accumulation reflecting NO released in the culture medium was quantified using the Griess reaction (*38*). Aliquots (100 μL) of cultured medium were dispensed into each well of a 96-well plate and mixed with 50 μL of 1% sulfanilamide solution (in 5% phosphoric acid). After 10 min of incubation at room temperature in the dark, 50 μL of 0.1% NED [*N*-(1-naphthyl)ethylenediamine dihydrochloride] solution was added to each well and thoroughly mixed. The absorbance at 540 nm was measured after 10 min of incubation at room temperature. Sodium nitrite ($NaNO_2$) solution prepared in DMEM was used to generate a standard curve. The nitrite concentrations of samples were determined by interpolation from the standard curve.

Prostaglandin E_2 (PGE_2) Determination. The PGE_2 level in the culture medium was quantified using a PGE_2 ELISA kit, (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instruction. In brief, aliquots (50 μL) of the cell medium were transferred into each well of a 96-well microtiter plate. After overnight incubation at 4 °C, the medium sample was removed and the plate washed with washing buffer four times. Each well was replenished with 200 μL of Ellman's reagent and incubated for an additional 1 h under ambient temperature. After incubation, the wells were emptied and washed with washing buffer six times followed by replenishment with 100 μL of avidin−peroxidase solution and incubation under ambient temperature for 1.5 h without exposure to light. The absorbance at 405 nm of each well was measured in a microplate spectrophotometer.

Pro-inflammatory Cytokine Quantification. Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) released by LPS-activated RAW 264.7 cells were quantified using ELISA kits (R&D System Inc., Minneapolis, MN) according to the manufacturer's instruction. Aliquots (50 μL) of the medium samples were dispensed into wells of a 96-well ELISA plate and incubated at ambient temperature for 2 h on a horizontal orbital shaker set at 500 rpm. The fluid was removed, and the wells were washed with washing buffer four times. Each well was replenished with 50 μL of detection mix included in the assay kit. The plate was firmly covered with a plate sealer and incubated for 2 h under ambient temperature on a shaker set at 500 rpm, followed by washing with buffer four times. After complete removal of liquid, 50 μL of streptavidin−horseradish peroxidase (HRP) was added to each well. The plate was incubated at ambient temperature for 30 min. The wells were washed one more time prior to the addition of 100 μL of tetramethylbenzidine (TMB) substrate solution. After 30 min incubation at ambient temperature in the dark, 50 μL of stop solution was added to cease further reaction. The absorbance at 450 nm of each well was detected using a microplate spectrophotometer.

RT-PCR Analysis. Expression of inflammatory gene on the messenger level was determined by reverse transcription Polymerase Chain Reaction (RT-PCR) analysis. Total RNA was isolated from the treated cells using TRIzol reagent (Invitrogen Co., San Diego, CA) according to the manufacturer's protocol. Three micrograms of the purified RNA was used in a 20 μL reaction volume for the first-strand cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen Co., San Diego, CA) with oligo$(dT)_{12\text{-}18}$ primer. One microliter of cDNA product was used as template for further PCR amplification using recombinant *Taq* DNA polymerase (Takara Bio Inc., Shiga, Japan). The primers targeting specific genes (**Table 1**) and thermal conditions conducted for amplification were adapted from previous reports (*39*−*44*). The PCR products were analyzed on 1.5% agarose gel and stained with ethidium bromide. The intensities of the PCR products on gel were quantified using Multi Gauge V3.0 (FujiFilm, Tokyo, Japan) after digital photography.

Statistical Analyses. Statistical analyses were conducted using SPSS for Windows version 10.0 (Statistical Program for Social Sciences, SPSS Corp., Chicago, IL). Analysis of variance (ANOVA) and Pearson's correlation coefficients were performed to compare the data. All determinations were performed in triplicate, and data are presented as mean ± SD. The confidence limits used in this study were based on 95% ($p < 0.05$).

Table 1. Sequence of Primers for RT-PCR

gene	primer sequence
β-actin	5′ TTCTTTGCAGCTCCTTCGTTGCCG 3′ 5′ TGGATGGCTACGTACATGGCTGGG 3′
TNF-α	5′ ATGAGCACAGAAAGCATGATC 3′ 5′ TACAGGCTTGTCACTCGAATT 3′
IL-6	5′ ATGAAGTTCCTCTCTGCAAGAGACT 3′ 5′ CACTAGGTTTGCCGAGTAGATCTC 3′
iNOS	5′ CTGGCAGCAGCGGCTCCATG 3′ 5′ GAAAAGACCGCACCGAAAGAT 3′
IL-1β	5′ CAGGATGAGGACATGAGCACC ′3 5′ CTCTGCAGACTCAAACTCCAC 3′
COX-2	5′ TGTATCCCCCCACAGTCAAAGACAC 3′ 5′ GTGCTCCCGAAGCCAGATGG 3′

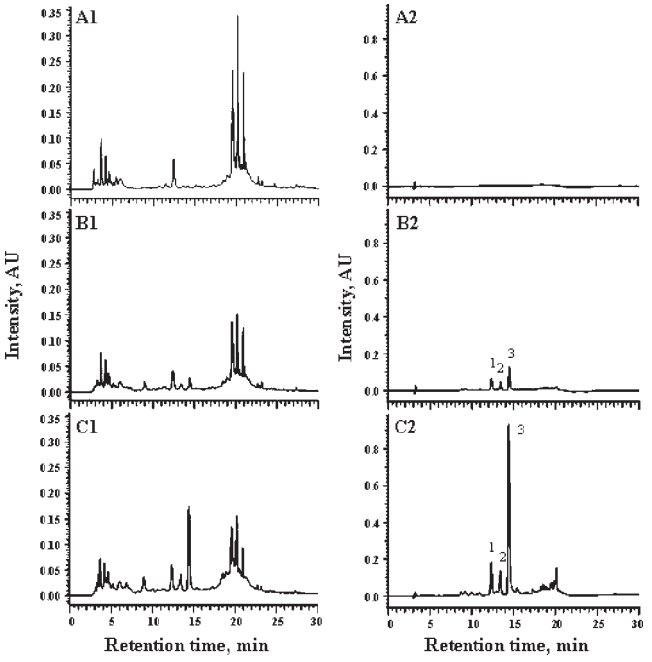

Figure 2. HPLC chromatograms monitored, respectively, at 280 nm (**A1**−**C1**) and 530 nm (**A2**−**C2**) of pigment extracts of *Basella alba* fruits at various maturities: (**A**) immature fruits; (**B**) partially mature fruits; (**C**) mature (ripe) fruits. Three red-violet pigments were separated and assigned as peaks 1, 2, and 3.

RESULTS AND DISCUSSION

Fruits Pigment Analysis at Various Maturities. *B. alba* fruits in bunches (**Figure 1C1**) were separated into three maturity sublots and subjected to pigment extraction and HPLC analysis. The chromatographs were monitored at 280 and 530 nm, with an attempt to detect flavonoids and red-colored compounds, as shown in **Figure 2**. As observed, substantial quantity of flavonoids in the immature (green-colored) fruits were detected between 19 and 21 min of retention time (**Figure 2A**). No peak was detected at 530 nm, indicating that red pigment was not present in the green and immature fruits. On the basis of the 530 nm monitored chromatograms of the extracts of partially and fully mature fruits, three well-resolved red pigments assigned as pigments 1, 2, and 3 were detected. As affected by maturity, it is obvious that the quantity of pigment 3 increased remarkably from partially mature fruits to mature fruits and that peak areas of the detected flavonoid peaks (19−21 min of retention time) decreased.

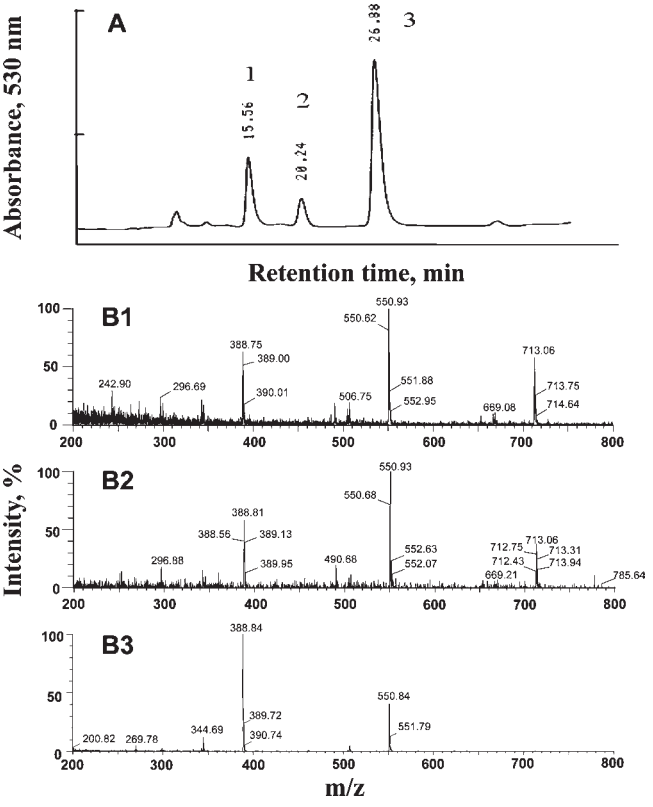

Figure 3. Semipreparative HPLC chromatogram monitored at 530 nm (**A**) of the red-violet pigments of *Basella alba* fruits subjected to mass spectrometric analysis to indicate the spectra of the collected substances under each of the peaks indicated with 1 (**B1**) betanidin-dihexose, 2 (**B2**) isobetanidin-dihexose, and 3 (**B3**) gomphrenin I (betanidin-hexose).

Pigment Isolation and Identification. Subjecting mature fruit flesh of *B. alba* to methanol extraction, C18 SPE column pretreatment and semipreparative HPLC analysis, the 530 nm monitored chromatogram (**Figure 3A**) indicated three major pigment peaks were well-resolved and similar to those detected by analytical HPLC (**Figure 2C2**). As analyzed by semipreparative HPLC, their retention times were 15.56, 20.24, and 26.88 min, respectively. On the basis of diode array UV−vis spectra (220−550 nm) (data not shown), two absorption maxima were located at 272 nm and between 530 and 540 nm. As reported (*1*), betacyanins display two absorption maxima, one in the UV range between 270 and 280 nm due to cyclo-dopa structure and another one in the visible range between 535 and 538 nm to indicate red color. Similar absorption spectra of betacyanins were also reported in purple Swiss chard, fruits of *Mammillaria*, and Mexican prickly pears analyzed by LC-DAD-ESI-MS-MS2 (*13*, *15*−*17*). Accordingly, the three detected pigments of *B. alba* fruits were most likely to be betacyanins.

Each of the pigment fractions was subjected to LC-MS analyses by a positive ion mode, and their MS spectra are shown in **Figure 3B**. As shown by MS spectra of both pigments 1 and 2 (**Figure 3B1**, **B2**), a protonated molecular ion at m/z 713 and two daughter ion fragments, respectively at m/z 551 and 389, indicating the presence of a dihexose moiety ($713 - 389 = 2 \times 162$), were detected. Thereafter, the pigments could be elucidated as isomers of betanidin-dihexose. This elucidation was supported by a previous study (*45*) showing that pigments 1 and 2 are C15 stereoisomers of betanidin-dihexose. For pigment 3 detected in the fruits of *B. alba*, a protonated molecular ion at m/z 551 and a daughter ion fragment at

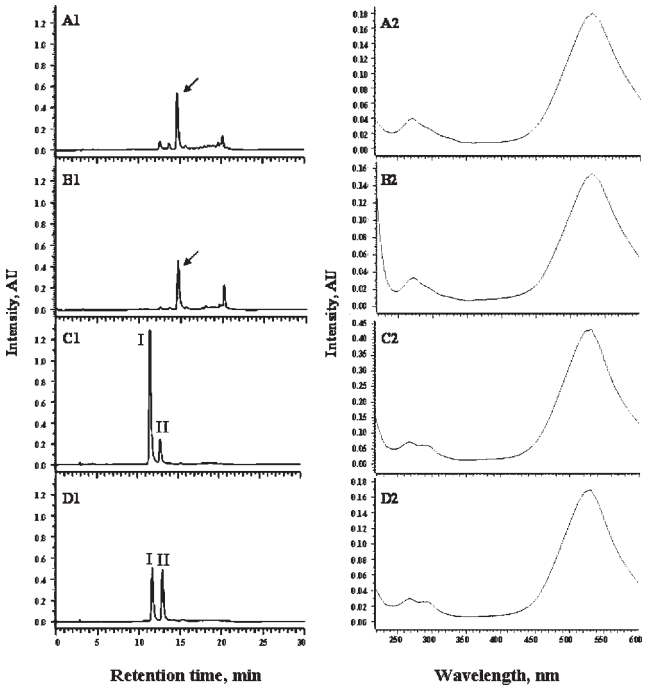

Figure 4. HPLC chromatograms (**A1**−**D1**) monitored at 530 nm and UV−vis spectra (**A2**−**D2**) of 80% methanol extracts of *Basella alba* fruits (**A**), *Basella rubra* fruits (**B**), and *Beta vulgaris* (**C**) and authentic betanin (**D**). The arrow indicates gomphrenin, betanin is indicated with I, and isobetanin is indicated with II.

m/*z* 389 were detected (**Figure 3B3**). Apparently, pigment 3 contained a hexose moiety (551 − 389 = 162) and could be elucidated as betanidn-hexose. This was in agreement with earlier papers on the extraction and structural elucidation of betalains from various natural sources (*2*, *10*, *15*, *17*, *45*).

Gomphrenin I Identification. As pigment 3 was the predominant colorant in *B. alba* fruits, its structure was further investigated. Betanin and isobetanin (15*S*-betanidin 5-*O*-*β*-glucoside) are the major pigments of *B. vulgaris* (sugar beet), which have been intensively investigated (*3*, *13*, *46*). Betacyanins from *B. rubra* fruits, a red-leaf variety, have been isolated and identified (*8*, *9*), in which gomphrenin I (15*S*-betanidin 6-*O*-*β*-glucoside) is the major betacyanin pigment and minor pigments are gomphrenin II [15*S*-betanidin 6-*O*-[6′-*O*-(4-coumaroyl)]-*β*-glucoside], isogomphrenin I (15*S*-betanidin 6-*O*-*β*-glucoside), and gomphrenin III (15*S*-betanidin-6-*O*-[6′-*O*-feruloyl-*β*-glucoside]). For further confirmation of the major pigment (peak 3) in *B. alba* fruits as gomphrenin I but not betanins, HPLC chromatograms and diode array UV−vis spectra of the major pigment extracts of *B. alba* and *B. rubra* fruits and roots of red sugar beets (*B. vulgaris*) and authentic betanins were compared (**Figure 4**). On the basis of the resultant identities of retention time and UV−vis spectra, pigment 3 of *B. alba* (**Figure 4A1**, **A2**) was identical to gomphrenin I of *B. rubra* (**Figure 4B1**, **B2**). This was supported by differentiation of the spectrum and the retention time of gomphrenin I from those of betanins extracted from *B. vulgaris* (**Figure 4C1**, **C2**) or authentic betanins (**Figure 4D1**, **D2**). The authentic betanin sample contained betanin and isobetanin (I and II indicated in **Figure 4D**) (15*S*-betanidin 5-*O*-*β*-glucoside) (*3*, *13*, *46*), which were also detected in *B. vulgaris* extract (**Figure 4C**). Thus, the major red-violet pigment elucidated as gomphrenin I (15*S*-betanidin 6-*O*-*β*-glucoside) was confirmed. As estimated, the yield of gomphrenin I recovered by the developed procedure was 36.1 mg/100 g of fresh fruits.

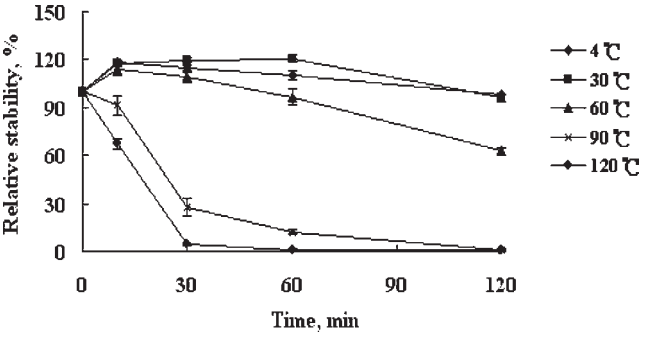

Figure 5. Thermal stability of gomphrenin I subjected to various heat treatments at 4, 30, 60, 90, and 120 °C for 0, 10, 30, 60, and 120 min.

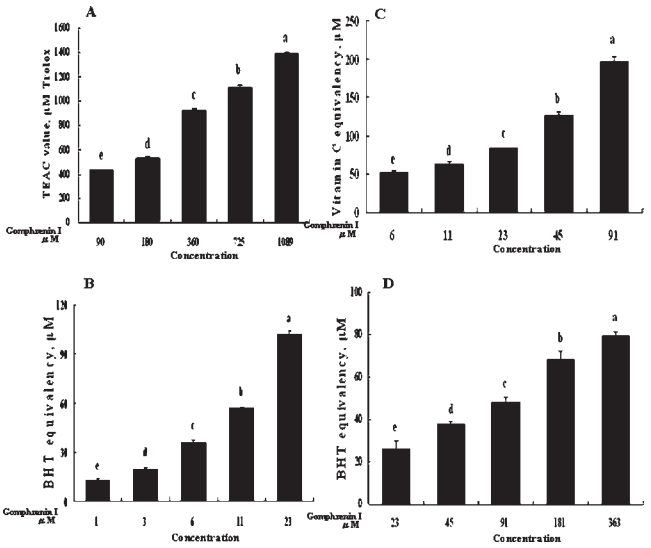

Figure 6. Antioxidant activities of gomphrenin I of *Basella alba* fruits: (**A**) Trolox equivalent antioxidant capacity (TEAC); (**B**) diphenylpicrylhydrazyl (DPPH) scavenging activity; (**C**) reducing power; (**D**) antioxidative potency. Each value represents the mean ± SD (n = 3). Bars with different letters are significantly different ($p < 0.05$).

Thermal Stability. As aqueous gomphrenin I solutions (25 μg/mL) were subjected to various temperatures for storage for 120 min, their absorbance at 530 nm (red color) at 90 and 120 °C decreased rapidly with an increase in storage time (**Figure 5**). At temperatures of 4, 30, and 60 °C, slight increases in absorbance at 530 nm were detected after 60 min of storage. At 60 °C, absorbance at 530 nm was stable for 60 min and approximately 75% of the absorbance was retained after 120 min of storage. From the viewpoint of exploring novel sources of natural colorants, the observed thermal stability of gomphrenin I destined as a natural colorant used in minimally processed foods, such as desserts, drinks, yogurts, and ice creams, has potential.

Antioxidant Activity. After the purified gomphrenin I had been subjected to antioxidant activity characterization, its activities, respectively assessed by Trolox equivalent antioxidant capacity (TEAC), DPPH scavenging activity, reducing power, and AOP, are shown in **Figure 6**. In each gomphrenin I concentration test range, the antioxidant activity increased with the increase in concentration in a dose-dependent manner. Compared to the referenced antioxidants, the detected TEAC value of gomphrenin I at 180 μM was equivalent to 534 μM Trolox in scavenging ABTS radical cations (**Figure 6A**). The scavenging activity of gomphrenin I at 23 μM was equivalent to 103 μM BHT in scavenging DPPH radicals (**Figure 6B**). Its reducing power at

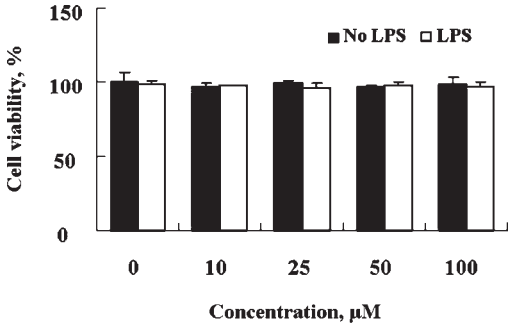

Figure 7. Cell viabilities of RAW 264.7 macrophage as affected by gomphrenin I of *Basella alba* fruits. Each value represents the mean ± SD ($n = 3$). Bars with different letters are significantly different ($p < 0.05$).

45 μM was equivalent to that of 129 μM vitamin C (**Figure 6C**). These results suggest that gomphrenin I purified from *B. alba* fruits possesses higher antiradical and antioxidative activities than do Trolox, BHT, and ascorbic acid. This finding is consistent with the study by Cai et al. (*21*) indicating that simple gomphrenin is the strongest antioxidant among betalains extracted from 37 species of Amaranthaceae plants and exhibits 3- to 4-fold stronger antioxidant activity than ascorbic acid. In this study, the AOP of gomphrenin I at 181 μM was equivalent to that of 68 μM BHT in the inhibition of linoleic acid peroxidation (**Figure 6D**). In comparison, and on the basis of the very hydrophilic nature of gomphrenin I, the low detected AOP might result from possible hindrance of interactions among gomphrenin I molecules and the emulsified substrate of linoleic acid.

Anti-inflammatory Function. Accumulating evidence suggests that inflammation plays a critical role in the pathogenesis of various types of human diseases, including cancers (*47*) and atherosclerosis (*48*). Great effort has been made to explore the dietary phytochemicals that can provide anti-inflammatory effects.

The anti-inflammatory activity of gomphrenin I purified from *B. alba* fruits was assessed in vitro addressed on its inhibitory effects on the production of inflammatory mediators, including NO, PGE_2, TNF-α, IL-1β, and IL-6 by RAW 264.7 cells stimulated with endotoxin, LPS. To assess the bioactivities of gomphrenin I without eliciting a cytotoxic effect, cells were cultured for 24 h in the LPS-free and LPS-introduced media containing 0, 25, 50, or 100 μM gomphrenin I. Exposure to gomphrenin I, regardless of dose and/or LPS stimulation, did not cause extra conversion of MTT to formazan (**Figure 7**). These indicated that gomphrenin I, at concentrations up to 100 μM, did not elicit cytotoxicity to the rest or activated macrophage cells. Accordingly, these doses of gomphrenin I were appropriate to be applied for anti-inflammatory assessments.

Inflammatory response is mediated by multiple factors including cytokines and noncytokines. NO and PGE_2 represent two major noncytokine mediators, which are biosynthesized by nitric oxide synthase (NOS) and cyclooxygenase (COX), respectively. Three major forms of NOS and COX each have been identified. Among those, inducible nitric oxide synthase (iNOS or NOS2) and cyclooxygenase-2 (COX-2) are usually involved in the up-regulation of inflammatory circumstances. NO was initially identified as a vascular relaxant, but is now considered as one of most versatile signaling molecules involved in various physiological homeostasis and pathophysiological processes (*49*).

As shown in **Figure 8**, RAW 264.7 cells stimulated with *Escherichia coli* LPS for 24 h significantly increased the nitrite and PGE_2 levels in the culture media. Introduction of gomphrenin I purified from *B. alba* fruit decreased LPS-induced NO

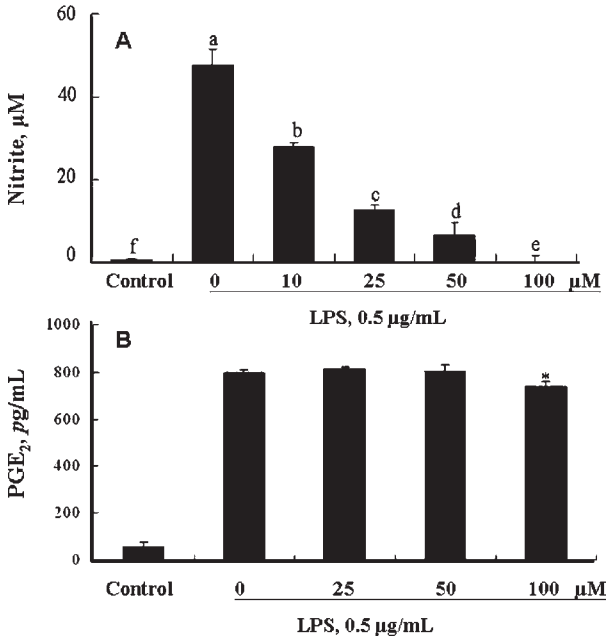

Figure 8. Nitric oxide (NO) and prostaglandin E_2 concentrations produced by lipopolysaccharide (LPS)-induced RAW 264.7 macrophage as affected by gomphrenin I of *Basella alba* fruits. Each value represents the mean ± SD ($n = 3$). Bars with different letters are significantly different ($p < 0.05$). Single asterisk (*), $p < 0.05$ versus LPS treatment only.

production in a dose-dependent manner (**Figure 8A**), and the half-maximal inhibitory concentration (IC_{50}) was 24.36 ± 3.35 μM. It is noteworthy that 100 μM gomphrenin I completely blocked NO production (**Figure 8A**). Significant inhibition on PGE_2 production was also observed in the cells treated with the highest concentration (100 μM) of gomphrenin I (**Figure 8B**).

In the immune response, NO plays a major role in killing phagocyted microbes. However, excessive NO production as a consequence of dysregulated induction of iNOS may generate peroxynitrite ($ONOO^-$), a potent reactive nitrooxidative species (RNS), by interaction with superoxide anion ($O_2^{\bullet -}$), leading to oxidative injury in the host. Prostaglandins are a class of eicosanoid lipid mediators derived from arachidonic acid via the catalytic action of COX. PGE_2 is the most abundant metabolite generated by COX-2 and is an inducer for vascular permeability, fever, and hyperalgesia. COX-2 is also a source of superoxide anion. Accordingly, COX-2 is the major target enzyme of nonsteroidal anti-inflammatory drugs (NSAIDs). Our finding that gomphrenin I decreased PGE_2 production associated with the inhibition of transcription expression of COX-2 (**Figure 9**) further confirmed the previous report that betalains isolated from *B. vulgaris* effectively inhibited COX-2 enzyme activity (*27*). In addition to iNOS and COX-2, myeloperoxidase (MPO) is a major enzyme that produces ROS in activated phagocytic leukocytes. Betanin and indicaxanthin have been detected exhibiting an inhibitory effect on MPO activity (*31*). The inhibition of ROS-generating enzymes should be counted in both anti-inflammatory and antioxidant properties of betalains.

TNF-α and IL-1β are classified as pro-inflammatory cytokines released mainly by macrophages at the initiation phase of inflammation. Both cytokines act on the endothelium of blood vessels adjacent to an infected site and induce expressions of cellular adhesion molecules that promote leukocyte extravasation. High quantities of TNF-α and IL-1β may cause systemic inflammatory response and pathologic abnormalities. Treatment of endothelial cells with betalains extracted from cactus pear

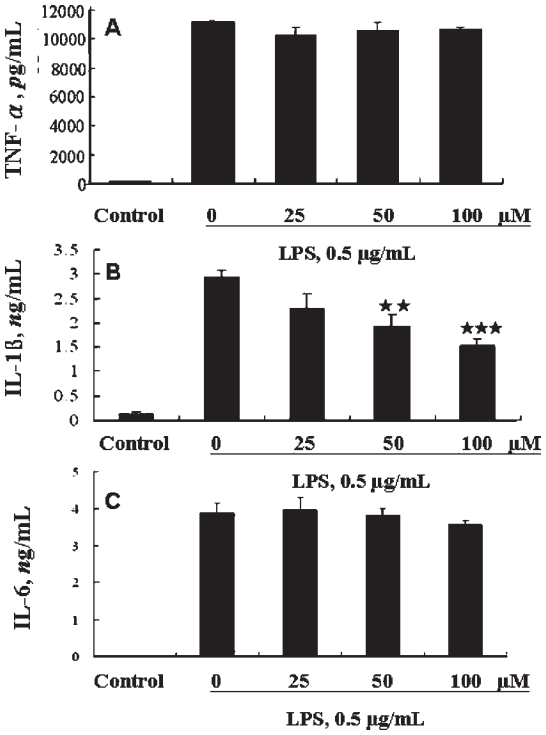

Figure 9. Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) concentrations produced by lipopolysaccharide (LPS)-induced RAW 264.7 macrophage as affected by introduction of gomphrenin I of *Basella alba* fruits. Each value represents the mean ± SD (n = 3). Double asterisk, $p < 0.05$; triple asterisk, $p < 0.01$.

resulted in inhibition of the expression of intercellular adhesion molecule-1 (ICAM-1), one of the TNF-α and IL-1β target genes in endothelial cells (*50*). In this study, gomphrenin I did not exhibit significant effect on LPS-induced TNF-α and IL-6 prodcution by the macrophage cells (**Figure 9A**, **C**). However, at higher concentrations, 50 or 100 μM, gomphrenin I markedly inhibited IL-1β secretion (**Figure 9B**). The selective inhibition of gomphrenin I on IL-1β secretion is of interest. TNF-α and IL-1β may exert a redundant effect in innate immunity; however, emerging studies suggest that IL-1β plays the major role in the pathogenesis of autoinflammatory diseases and recurrent inflammatory diseases, such as gout, atherosclerosis, and type 2 diabetes (*51*−*53*). IL-1β is synthesized as a p35 precursor that must be cleaved to active form (p17) by IL-1β conversing enzyme (ICE), also known as caspase-1, before it is released into extracellular environment. Caspase-1 is categorized as an inflammatory caspase and activated by a large multiprotein complex termed inflammasome. NLRP3 inflammasome, formed by Nod-like receptor protein NLRP3, caspase recruitment domain-containing protein CARDINAL, adpator protein ASC, and caspase-1, has a vital role affecting the secretion of IL-1β by monocytes and macrophages in response to pathogenic microbe or host-derived signals of cellular stress (*54*, *55*). Specific targeting of NLRP3 inflammasome and selective inhibition of IL-1β secretion are new therapeutic approaches for recurrent inflammatory diseases such as gout (*56*). Accordingly, it is intriguing to know whether the specific inhibition of IL-1β secretion by gomphrenin I is via modulation of NLRP3 inflammasome.

In addition to regulation at secretion, cytokines and inflammatory mediators are also controlled at the transcriptional level. Macrophages in resting state, that is, without LPS stimulation, exhibited limited expression in inflammatory genes encoding TNF-α, IL-1β, IL-6, iNOS, and COX-2. As revealed by

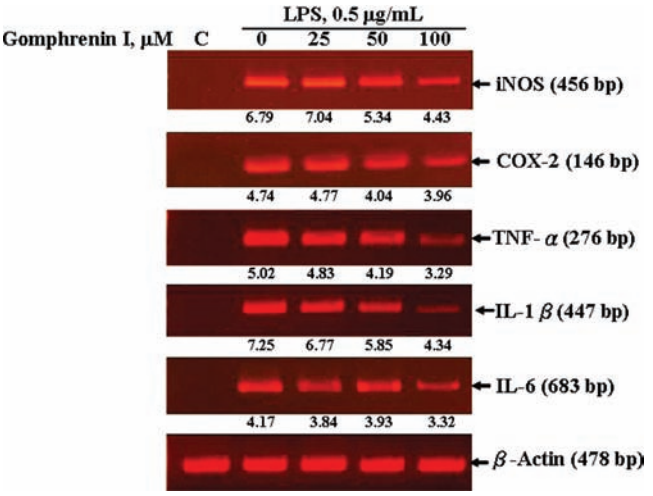

Figure 10. Inflammatory gene expression in lipopolysaccharide (LPS)-induced RAW 264.7 macrophage as affected by introduction of gomphrenin I of *Basella alba* fruits. Cells were cultured in the medium containing 0.5 μg/mL LPS only or with various concentrations of gomphrenin I purified from *B. alba* fruits for 24 h. Expression of inflammatory genes was analyzed by RT-PCR. The values under the image indicate relative intensity of PCR products normalized to β-actin.

RT-PCR detection (**Figure 10**), a high dose (100 μM) of gomphrenin I significantly inhibited LPS-induced expression of all inflammatory genes tested. This result suggests the possibility that gomphrenin I targets common transcription factors of those inflammatory genes. It is well established that LPS activates monocytes and macrophage mainly through the signaling ignited by transmembrane toll-like receptor 4 (TLR4). The binding of LPS to TLR4 conveys several intracellular signaling pathways in association with the activation of a variety of transcription factors and subsequently regulates inflammatory gene expression (*57*). Nuclear factor κB (NFκB) is considered to be a ubiquitous transcription factor for inflammatory mediators and is required for the induction of all LPS-inducible genes, including TNF-α, IL-1β, IL-6, NOS2, and COX2 (*57*). NFκB has also been postulated as a linking factor of inflammation to cancer development and progression (*58*). It is worthwhile to verify the effect and mechanism of gomphrenin I on NFκB activation. Further studies extended to intensive investigations into the food toxicology and bioavailability of gomphrenin I are needed. However, this is the first study, to the best of our knowledge, to demonstrate the anti-inflammatory activity of gomphrenin I through the inhibition of LPS-induced NO, PGE_2, and IL-1β production and the gene expression of TNF-α, IL-1β, IL-6, iNOS, and COX-2, which marks gomphrenin I as a potential anti-inflammatory agent.

In conclusion, three red-violet pigments were detected in ripe *B. alba* fruits. Gomphrenin I was identified as the major pigment, and its quantity increased with the increase of fruit maturity. In addition to gomphrenin I, betanidin-dihexose and isobetanidin-dihexose were also detected. On the basis of the estimated yield of gomphrenin I up to 36.1 mg/100 g of fresh fruits, *B. alba* fruits could be regarded as a potent source of natural colorant. Its solutions were stable at 60 °C but unstable when subjected to heat treatment at temperatures of 90 and 120 °C, indicating that it could be used in dry state or minimally processed foods. Compared to the referenced antioxidants (Trolox, ascorbic acid, and BHT), gomphrenin I could be classified as a very potent natural antioxidant. Gomphresnin I was also a potential anti-inflammatory agent that exhibited potent inhibitory activities against endotoxin-induced NO, PGE_2, and IL-1β secretion

and transcriptional expression of inflammatory genes encoding iNOS, COX-2, TNF-α, IL-1β, and IL-6. This confirms *B. alba* fruits as a rich source of betalain pigments, antioxidants, and inflammatory inhibitors and promotes *B. alba* fruits' value-added potential for use in the development of food colorants, nutraceuticals, and pharmaceuticals to benefit health and prevent diseases.

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