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Characterisation of the pigment components in red cabbage (*Brassica oleracea* L. var.) juice and their anti-inflammatory effects on LPS-stimulated murine splenocytes

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Abstract

To determine anti-inflammatory effects of pigments from red cabbage, red cabbage (*Brassica oleracea* L. var.) juice was prepared, characterized by UV–vis absorption spectra, partially purified by Sephadex LH-20 column, analyzed by HPLC, and administered to lipopolysaccharide (LPS)-stimulated murine splenocyte cultures. The study showed that red cabbage juice (RC) exhibited anti-inflammatory effects against LPS-induced inflammation of splenocytes via increasing anti-inflammatory cytokine interleukin (IL)-10 and decreasing pro-inflammatory cytokine IL-6 secretions. The maximum absorption peaks of RC and its heated products, but not activated charcoal-adsorbed products, appeared at 280 nm with a small shoulder around 310–330 nm while there existed a minor peak at 560 nm (range from 480 to 630 nm), reflecting red cabbage juice included phenolics, flavonoids, and anthocyanins. The lyophilized powder of chromatographic fractions F2, F3, and F4 through Sephadex LH-20 column were rich in phenolics ($5.9 \pm 0.2\%$, $4.4 \pm 0.0\%$, and $3.9 \pm 0.0\%$, respectively) and flavonoids ($1.8 \pm 0.3\%$, $1.8 \pm 0.3\%$, and $1.1 \pm 0.3\%$, respectively). The results suggest that anti-inflammatory pigment compounds in red cabbage juice were heat stable. Further analysis of chromatograms from HPLC suggests malvidin glycosides including malvidin 3-glucoside (oenin), malvidin 5-glucoside and malvidin 3,5-diglucoside in red cabbage juice could inhibit IL-6 secretion of LPS-stimulated splenocytes.

Keywords: Red cabbage; *Brassica oleracea* L.; Phenolics; Flavonoids; Anthocyanins; Malvidin; Interleukin (IL)-6; IL-10; Lipopolysaccharide (LPS)-stimulated splenocytes

1. Introduction

Inflammation can be defined by the sequential release of mediators such as pro-inflammatory cytokines, including interleukin (IL)-1, tumor necrosis factor (TNF), interferon (IFN)-γ, IL-6, IL-12, IL-18, and the granulocyte-macrophage colony-stimulating factor. Excess inflammation status can be controlled by anti-inflammatory cytokines such as IL-4, IL-10, IL-13, IFN-α, and the transforming growth factor (TGF)-β (Hanada & Yoshimura, 2002). Inflammation is overall a protective response against xenobiotics such as bacteria, virus, and parasites. However, chronic and uncontrolled inflammation is detrimental to tissues (Gil, 2002), which may cause chronic inflammation-derived diseases, such as cardiovascular diseases (Frostegard et al., 1999), autoimmune rheumatoid arthritis (RA) (Jara, Medina, Vera-Lastra, & Amigo, 2006), systemic lupus erythematosus (SLE), cancers (Karin, Lawrence, & Nizet, 2006), and aging-associated diseases, such as Alzheimer's or Parkinson's disease (Sarkar & Fisher, 2006). Inhibition of the overproduction of inflammatory mediators such as pro-inflammatory cytokines IL-1β, IL-6, and TNF-α (Kim et al., 2003) and increase of the secretion of anti-inflammatory cytokine IL-10 (Li, Guo, & Yang, 2005) by immune effector cells may prevent a variety

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of inflammation-derived diseases. A negative correlation between the consumption of diets rich in fruits, and vegetables and the risks for chronic diseases, such as cardiovascular diseases, arthritis, chronic inflammation and cancers exists (Chen et al., 2006; Prior, 2003; Saleem, Husheem, Harkonen, & Pihlaja, 2002; Zhang, Vareed, & Nair, 2005). There has been a growing interest in pigment components from fruits and vegetables which may promote human health or lower the disease risk (Lin & Tang, 2007, 2008a). Due to the attractive colours and higher contents of antioxidant phytochemicals (Singh et al., 2006), we hypothesized that red cabbage (*Brassica oleracea* L. var.) has potential in anti-inflammation.

Cabbage, which belongs to the family Cruciferae as well as the cultivated cabbage which is categorized into white cabbage, red cabbage and savoy cabbage, is one of the most important vegetables grown worldwide (Singh et al., 2006). Recently, red cabbage has attracted much attention because of its physiological functions and applications. The anthocyanins from red cabbage are stable under the acidic gastric digestion conditions, although all of the anthocyanins are reduced in content after pancreatic digestion but acylated forms were notably more stable than non-acylated forms (McDougall, Fyffe, Dobson, & Stewart, 2007). Thus, red cabbage dye has been used as a pH indicator in pharmaceutical formulations (Chigurupati, Saiki, Gayser, & Dash, 2002) and as a colorant in food systems (Giusti & Wrolstad, 2003). Anthocyanins in red cabbage inhibit amyloid β protein-induced neurotoxicity in neuron-like PC12 cells (Heo & Lee, 2006). Red cabbage juice via gavage to ICR female mice displayed a protective effect on oxidative stress in brain of mice administered i.p. with *N*-methyl-D-aspartate (NMDA) (Lee, Sok, Kim, & Kim, 2002).

The most important natural pigments are carotenoids, tetrapyrrole derivatives, and flavonoids in plants (Merken & Beecher, 2000). Dietary flavonoids, which are one class of plant polyphenols, are usually glycosylated and can be classified as anthocyanidins, flavanols (catechins), flavones, flavanones, and flavonols which are responsible for the orange, red and blue colours in fruits and vegetables (Lin & Tang, 2007; Merken & Beecher, 2000). Red cabbage (*B. oleracea*) leaves contain cyanidin 3,5-diglucoside, cyanidin 3-sophoroside-5-glucoside, cyanidin 3-sophoroside-5-glucoside and cyanidin-3-sophoroside-5-glucoside acylated with 1 and 2 mol of sinapic acid (Tanchev & Timberlake, 1969). Anthocyanidins, delphinidin and cyanidin but not pelargonidin, peonidin and malvidin, inhibit cyclooxygenase-2 (COX-2) expression, in lipopolysaccharide (LPS)-activated murine macrophage RAW264 (Hou, Yanagita, Uto, Masuzaki, & Fujii, 2005). COX-2 plays an important role in inflammation and tumorigenesis. Red cabbage is rich in anthocyanidins (Tanchev & Timberlake, 1969), suggesting that red cabbage juice may have anti-inflammatory potential. However, the study on anti-inflammatory pigment components of red cabbage is still limited.

This aim of the present work was to characterize pigment components in red cabbage (*B. oleracea* L. var.), to evaluate the anti-inflammatory effects on LPS-stimulated murine splenocytes, and to find the relationships among the pigment components in red cabbage juice and their anti-inflammatory effects on LPS-stimulated splenocytes.

2. Materials and methods

2.1. Preparation of red cabbage juice

The red cabbage of *B. oleracea* L. var. was purchased from a local supermarket in Taichung, Taiwan, in December, 2003. The fresh sample was immediately, without storage, squeezed to juice by a manual stainless screw squeezer (Vegetable and Fruit Grinder, manual type, Mei-Er-Then Co., Ltd., Taipei, Taiwan). The juice was centrifuged at 9000*g* (4 °C) for 30 min, and then the supernatant was collected using suction filtration through filter papers (Toyo No. 5B, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The extraction efficiency of red cabbage juice was 69.1%. The filtrate was measured, lyophilized, and stored at −30 °C for future use (Lin & Tang, 2007). The yield of lyophilized powder from the red cabbage juice was 5.3%. The lyophilized powder of crude red cabbage juice is referred to as RC. Heat may destroy most immunological activities of the proteinaceous substances *in vitro*. To minimize the interference of protein in RC, a part of RC was dissolved in deionized water, heated at 100 °C for 20 min and then lyophilized to harvest the heat-treated sample powder (HRC). Activated charcoal can adsorb low-molecular-weight components such as dyes (Iqbal & Ashiq, 2007). To eliminate the pigment in RC, a part of RC powder was dissolved in deionized water and then slowly mixed with activated charcoal (1:1, w/w) to adsorb small molecules. After standing at 4 °C for 30 min, the mixture was filtered through a filter paper (Toyo No. 5B, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) to obtain activated charcoal-adsorbed juice. The supernatant was lyophilized to obtain an activated charcoal-treated sample (RCA). The recovery of RCA was 65 ± 3%. A part of RC was re-dissolved and subjected to size-exclusion chromatography using Sephadex LH-20 column to further separate the possibly bioactive pigment components (Roybal, Pfenning, Turnipseed, & Gonzales, 2003). The UV–vis absorption spectra of RC and its treated samples were recorded (Sousa et al., 2007).

2.2. Chromatographic fractionation of red cabbage juice

2.2.1. Sephadex LH-20 column chromatography

2.2.1.1. Sephadex LH-20 column preparation. Twenty grams of dry Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden) was mixed with 80 mL of methanol (Mallinckrodt Chemicals, Phillipsburg, NJ, USA) for 3 h to activate the gel. The activated gel/methanol (3:1, v/v) was carefully poured into a glass chromatographic column (15 mm i.d. × 370 mm). The column walls were washed with 10 mL of methanol. The column was allowed to stand

at room temperature for Sephadex gel to settle. To equilibrate the column, the column was drained and washed with methanol for 1 h before use (Roybal et al., 2003).

2.2.1.2. Isolation and purification of pigment components in red cabbage juice using Sephadex LH-20 column. About 2 g of RC was dissolved in 10 mL deionized water, then separated (1 mL) on the Sephadex LH-20 gel filtration column at a flow of 0.5 mL/min controlled by peristaltic pump (Iwaki pst-110). Elution was carried out with 90 mL of 100% methanol, followed by 60 mL of 50% methanol/water (v/v) and finally with 60 mL of water. The eluent (3 mL/tube) was collected using a fraction collector (Isco Retriever 500, Teledyne Technologies, Inc., Lincoln, NE, USA) and detected at 254 or 280 nm using a spectrophotometer (Hitachi Model 100-20, Hitachi, Ltd., Tokyo, Japan). As Fig. 1a shown, these collected fractions are referred to as fraction 1 (F1, tube nos. 1–10, 30 mL), F2 (tube nos. 11–40, 90 mL), F3 (tube nos. 41–48, 24 mL), F4 (tube nos. 49–54, 15 mL), and F5 (tube nos. 55–61, 21 mL) and were concentrated using a rotary vacuum evaporator (Eyela Model 81-1, Tokyo Rikakika Co. Ltd., Tokyo, Japan) and then lyophilized using a freeze dryer (Panchum CT-5000D, Panchum Scientific Corp., Kaohsiung, Taiwan, ROC). This fractionation process was repeated several times and each individual fraction was pooled together. The yields of individual fractions (F1–F5) were 0.2%, 39.0%, 1.3%, 2.1%, and 0.4%, respectively. A portion of each fraction was heated at 100 °C for 20 min and then lyophilized to procure the heat-treated fraction powder (HF1–HF5). Fractions were collected separately for anti-inflammatory activity evaluation and bioactive component identification. The total phenolic and flavonoid contents in F1–F5 and HF1–HF5 were determined. At a later stage, a portion of lyophilized fractions (aliquots of 0.05 g) was re-suspended in 1 mL of 1% hydrochloric acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in methanol (HPLC-grade, Tedia Co. Inc., Fairfield, OH, USA) for 4 h at 4 °C, centrifuged at 4000*g* (4 °C) for 15 min, and then the supernatant was collected. The

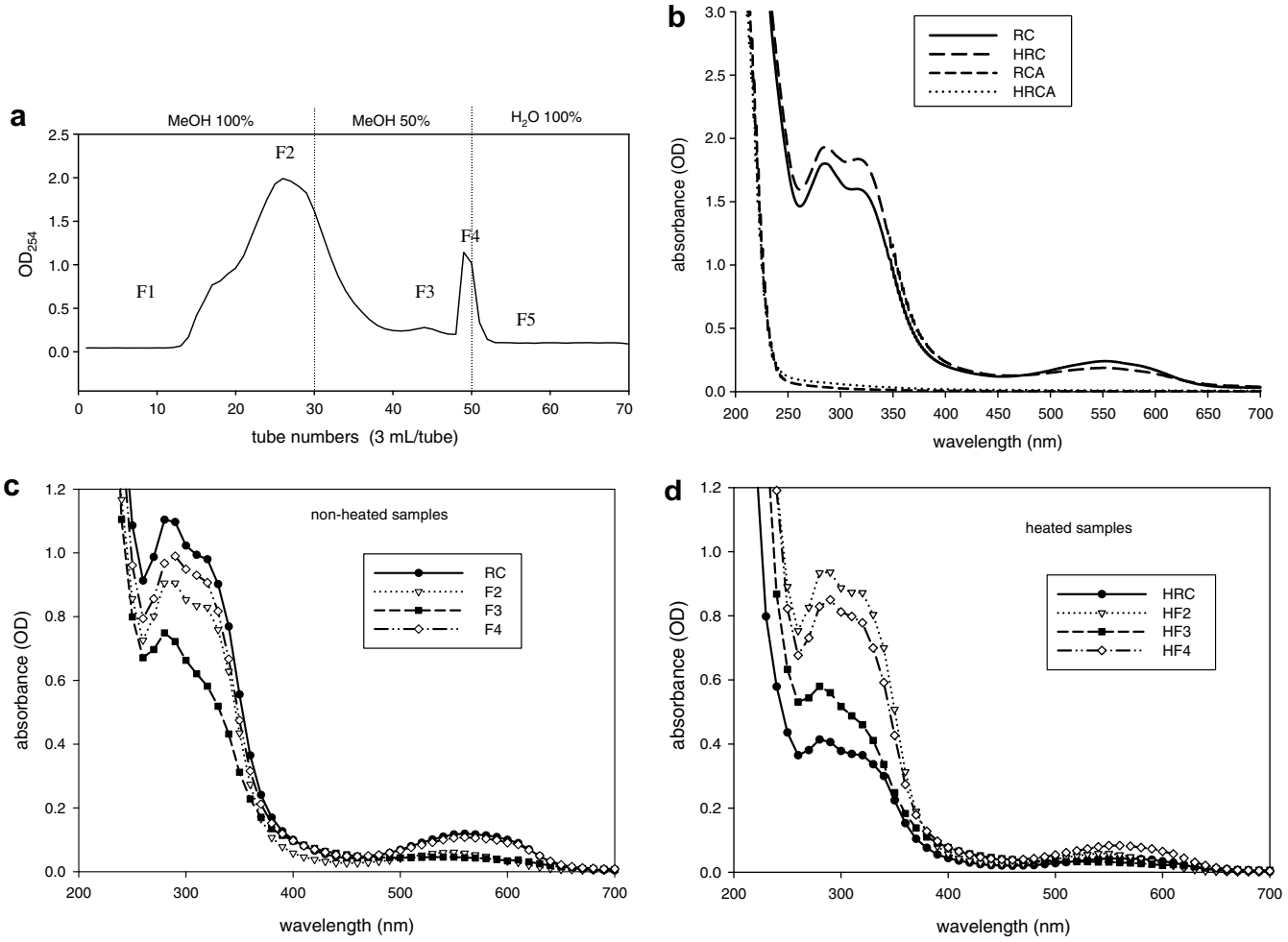

Fig. 1. Chromatographic profiles and selected chromatographic fractions of red cabbage juice through Sephadex LH-20 column detected by the absorbance at the wavelength of 254 nm (a), and UV–vis spectra of crude red cabbage juice as well as their different treated products (b–d). RC: red cabbage juice; HRC: heated RC; RCA: activated charcoal-adsorbed red cabbage juice; HRCA: heated RCA; Fs: chromatographic fractions; HFs: heated chromatographic fractions.

residue was re-treated with 1 mL of 1% hydrochloric acid in methanol and finally the supernatant was collected again. The total collected supernatant of individual fraction was filtered through a 0.45 μm filter membrane (Sartorius Minisart SRP 4, Vivascience AG, Hannover, Germany). The filtrate was stored at −30 °C for HPLC analysis (Chirinos et al., 2008; Yu et al., 2006).

2.2.2. HPLC analysis of chromatographic fractions of red cabbage juice through Sephadex LH-20 column

The chromatographic fractions (F1–F5) of red cabbage juice were separated by HPLC (Hitachi, pump: L-2131, detector: L-2400, interface: IFB) on a Mightsil RP-18 GP250-4.6 column (4.6 mm × 250 mm, particle size, 5 μm, Kanto Chemical Co. Inc., Tokyo, Japan) at a flow rate of 1 mL/min. The mobile phase was water/formic acid (9:1, v/v) as eluent A and water/formic acid/acetonitrile/methanol (40:10:22.5:22.5, v/v/v/v) as eluent B. The absorbance of the eluent was monitored at 530 nm. The HPLC chromatographic column was conditioned with 80% of eluent A and 20% of eluent B. After 20 μL of sample was injected into the column, the column was immediately eluted with 80% of eluent A and 20% of eluent B for 15 min and with the following increasing eluent B concentrations: 0–15 min, 20% of eluent B (v/v), 15–60 min, 25% of eluent B (v/v), 60–80 min, 40% of eluent B (v/v), 80–90 min, 80% of eluent B (v/v). Each fraction was analyzed in triplicate. Phenolic or anthocyanin compounds were, respectively, identified and qualified by comparing their retention time and UV–vis spectral data to known previously injected standards (Kader, Rovel, Girardin, & Metche, 1995; Yu et al., 2006).

2.3. Determination of total phenolic and flavonoid contents

Total phenolic contents in samples were determined using the Folin–Ciocalteu method (Meda, Lamien, Romito, Millogo, & Nacoulma, 2005; Zin, Hamid, Osman, & Sarri, 2006). Briefly, aliquots of 0.1 g lyophilized powder samples were, respectively, dissolved in 1 mL deionized water. This solution (0.1 mL) was mixed with 2.8 mL of deionized water, 2 mL of 2% sodium carbonate (Na_2CO_3, Wako Pure Chemical Industries, Ltd., Osaka, Japan), and 0.1 mL of 50% Folin–Ciocalteau reagent. After incubation at room temperature for 30 min, the reaction mixture absorbance was measured at 750 nm against a deionized water blank. Gallic acid (GA, Sigma–Aldrich Co., China), a major phenolic compound in red cabbage (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004), was chosen as a standard. Using a seven point standard curve (0–2000 mg/L), the levels of total phenolic contents in samples were determined in triplicate, respectively. The data were expressed as gram (g) gallic acid equivalents (GAE)/100 g lyophilized powder (Heimler, Vignolini, Dini, Vincieri, & Romani, 2006; Lin & Tang, 2007).

The total flavonoid content was determined according as the 2,4-dinitrophenylhydrazine (DNP) colorimetric method described by Chang, Yang, Wen, and Chern (2002). Briefly, aliquots of 0.02 g of samples were, respectively, dissolved in 0.6 mL of deionized water. This solution was mixed with an aliquot of 0.4 mL of DNP (Sigma–Aldrich Co., St. Louis, MO, USA) solution (an aliquot of 0.5 g of DNP in 50 mL of 50% methanol). After incubation at 50 °C for 50 min, an aliquot of 1 mL of potassium hydroxide (KOH, Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution (an aliquot of 0.5 g of KOH in 50 mL of 50% methanol) was added to the reactant mixture. Stand for 2 min, an aliquot of 8 mL of 50% methanol was added, centrifuged at 1000*g* for 15 min, and then the supernatant was collected. The reaction mixture absorbance was measured at 494 nm against a deionized water blank. Naringenin (Sigma–Aldrich Co., St. Louis, MO, USA), a main flavonoid component in red cabbage (Manach et al., 2004; Saleh, Poulton, & Grisebach, 1976), was chosen as a standard. Using a seven point standard curve (0–2000 mg/L), the levels of total flavonoid contents in samples were determined in triplicate, respectively. The data were expressed as g naringenin equivalents (NE)/100 g lyophilized powder.

2.4. Preparation of splenocyte cultures

The female BALB/c mice (6 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, Taiwan, ROC and maintained in the Department of Food Science and Biotechnology at National Chung Hsing University, College of Agriculture and Natural Resources in Taichung, Taiwan, ROC. The mice were housed and kept on a chow diet (laboratory standard diet). After the mice acclimatized for 2 weeks, the animals were weighed, anaesthetized with ether and immediately bled using retro-orbital venous plexus puncture to collect blood. Immediately after blood collection, the animals were sacrificed using CO_2 inhalation for primary splenocyte culture studies. The splenocytes were prepared by aseptically removing spleens from BALB/c mice. Spleens were homogenized in TCM (tissue culture medium, a defined commercial serum replacement, Celox Laboratories Inc., Lake Zurich, IL, USA) medium (10 mL of TCM, 500 mL of RPMI 1640 medium (Atlanta Biologicals, Inc., Norcross, GA, USA), and 2.5 mL of antibiotic–antimycotic solution (100×) containing 10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of amphotericin B in 0.85% saline (Atlanta Biologicals, Inc., Norcross, GA, USA). Single spleen cells were collected and treated by lysing the red blood cells with RBC lysis buffer (0.017 M Trizma base (Sigma–Aldrich Co., St. Louis, MO, USA), 0.144 M ammonium chloride (Sigma–Aldrich Co., St. Louis, MO, USA), pH 7.2, 0.2 μm filtered). Splenocytes were isolated from each animal and adjusted to 1×10^7 cells/mL in TCM medium with a hemocytometer using the trypan blue dye exclusion method. The splenocytes (0.50 mL/well) in the absence or presence of mitogens

(0.50 mL/well), such as lipopolysaccharide (LPS, 10 μg/mL, Sigma–Aldrich Co., St. Louis, MO, USA,) only, or both LPS and red cabbage juice products. The endotoxin LPS was used to induce inflammation of splenocytes (Lin, Lai, Liu, & Wu, 2007). To evaluate the anti-inflammatory potential of red cabbage juice products, an inflammation-concurrent cell culture model was designed using addition of LPS and test samples together (Lin & Tang, 2008b). The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for 48 h. The plates were centrifuged at 200*g* for 10 min. The supernatants in cell cultures were collected and stored at −70 °C for cytokine assays.

2.5. Measurement of pro-inflammatory and anti-inflammatory cytokine levels secreted by splenocyte cultures by an enzyme-linked immunosorbent assay (ELISA)

Pro-inflammatory cytokine (IL-1β, IL-6, and TNF-α) and anti-inflammatory cytokine (IL-10) levels were determined using sandwich ELISA kits, respectively. The IL-1β, IL-6, IL-10, and TNF-α concentrations were assayed according to the cytokine ELISA protocol from the manufacturer's instructions (mouse DuoSet ELISA Development system, R&D Systems, Minneapolis, MN, USA). The sensitivity of these cytokine assays was 15.6 pg/mL. One hundred microlitres of 1:180 diluted (with PBS) anti-mouse captured antibodies was added to 96-microwell plate wells (Nunc) and incubated overnight at 4 °C. After incubation, plates were washed four times with ELISA wash buffer (0.05% Tween 20 in PBS, pH 7.2–7.4). To block non-specific binding, 200 μL of block buffer (1% Bovine serum albumin (BSA), 5% sucrose in PBS with 0.05% NaN_3) were added to each well. The plates were incubated at room temperature for 1 h. After incubation, plates were washed three times with ELISA wash buffer. Volumes of 100 μL of BALF or standard in reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered Saline (20 mM Trizma base, 150 mM NaCl), pH 7.2–7.4, 0.2 μm filtered) were added to the 96-microwell plate wells and the plates were incubated at room temperature for 2 h. A seven point (in duplicate) standard curve using 2-fold serial dilutions in reagent diluent, and a high standard of 1000 pg/mL were conducted. After incubation, plates were washed four times with ELISA washing solution. One hundred microlitres of detection antibody (biotinylated goat antimouse monoclonal antibody at 1:180 dilution in reagent diluent) was then added to each well. Plates were incubated at room temperature for 2 h. After incubation, plates were washed six times with ELISA wash buffer. One hundred microlitres of working Streptavidin–HRP (horseradish peroxidase) dilution was added to each well. Plates were incubated at room temperature for 20 min. After incubation, plates were washed six times with ELISA washing buffer. One hundred microlitres of substrate solution (tetramethylbenzidine; TMB) was pipeted into the 96-microwell plate wells. Plates were incubated at room temperature for 20 min to develop colour. Fifty microlitres of stop solution (2 N H_2SO_4) was added to each well to stop the reaction. The plates were measured for absorbance at 450 nm on a plate reader (ELISA reader, ASYS Hitech GmbH, Eugendorf, Austria). Using the seven point (1000, 500, 250, 125, 62.5, 31.25, and 15.6 pg/mL) standard curves, the levels of cytokines in splenocyte cultures were determined, respectively (Lin et al., 2007).

2.6. Statistical analysis

Data are expressed as mean ± SD ($n = 3$) and analyzed statistically using ANOVA followed, if justified by the statistical probability ($P < 0.05$), by Dunnett's test of parametric type or Duncan's New Multiple Range test. Differences between the control and other treatments were considered statistically significant if $P < 0.05$.

3. Results

3.1. UV–vis absorption spectra of red cabbage juice

Fig. 1b shows the UV–vis absorption spectra of red cabbage juice, whether heated or not, and its activated charcoal-adsorbed products. It was found that the maximum absorption peaks of crude red cabbage juice (RC) and its heated product (HRC) appeared at 280 nm with a small shoulder around 310–330 nm while existed a minor peak at 560 nm (range from 480 to 630 nm). Heating did not decrease the absorbance of red cabbage juice. However, heat might result in water loss of red cabbage juice and slightly increased the absorbance. The absorption spectra reflected that pigments in red cabbage juice might consist of phenolics (280 nm), flavonoids (310–330 nm) and anthocyanins (480–630 nm). However, the activated charcoal adsorbed small molecular pigments in red cabbage juice and caused the disappearance of absorption peaks in the UV–vis spectra (Fig. 1b).

To further confirm the property of partially purified pigments in red cabbage juice, UV–vis spectra of non-heated or heated chromatographic fractions (F2–F4) of red cabbage juice through Sephadex LH-20 column were determined. The partially purified pigments, whether heated or not, exhibited an absorption peak at 280 nm with a shoulder around 310–330 nm while existed a minor peak at 560 nm (range from 480 to 630 nm) (Fig. 1c and d) which are similar to those of RC and HRC. The results from the absorption spectra of partially purified chromatographic fractions further reflected that pigments in red cabbage juice included phenolics (280 nm), flavonoids (310–330 nm), and anthocyanins (480–630 nm).

3.2. Effects of administrations with red cabbage juice and its chromatographic fractions on LPS-stimulated splenocytes

To examine anti-inflammatory potential of red cabbage juice (RC) and its chromatographic fractions, whether

heating or not, on LPS-stimulated murine splenocytes *in vitro*, red cabbage juice samples were, respectively, administered to LPS-stimulated splenocyte cultures for 48 h. Secretions of pro-inflammatory cytokines IL-1β, IL-6, as well as TNF-α, and anti-inflammatory cytokine IL-10 by LPS-stimulated splenocytes were determined. Table 1 shows the effects of RC administrations on pro- and anti-inflammatory cytokine secretions by LPS-stimulated splenocytes. The results showed that RC (50–1250 μg/mL) administrations significantly ($P < 0.05$) inhibited IL-6 secretion by LPS-stimulated splenocytes in a dose-dependent manner, while in the meantime markedly ($P < 0.05$) increased anti-inflammatory cytokine IL-10 secretion. However, RC did not significantly affect the secretions of IL-1β and TNF-α. The results suggested that red cabbage juice exhibited an anti-inflammatory effect against LPS-induced inflammation of primary splenocytes via increasing anti-inflammatory cytokine IL-10 and decreasing pro-inflammatory cytokine IL-6 secretions. Chromatographic fractions (F1–F5), whether heating or not, from red cabbage juice were further conducted to test their effects on the secretions of IL-6 and IL-10 by LPS-stimulated splenocytes. Table 2 shows the effects of RC and its chromatographic fraction (F1–F5) administrations on IL-6 and IL-10 secretions by LPS-stimulated splenocytes. The results showed that RC, F1, F2, F3 and F4 administrations significantly ($P < 0.05$) inhibited IL-6 secretion by LPS-stimulated splenocytes. F1, F2, and F3 (20–500 μg/mL) inhibited IL-6 secretion in a dose-dependent manner. Even though the samples treated with heating, HRC, HF1, HF2, HF3, and HF4 still demonstrated strong activity against IL-6 secretion (Table 2). Table 2 also shows the effects of RC and its chromatographic fraction (F1–F5) administrations on IL-10 secretions by LPS-stimulated splenocytes. The results showed that RC, F1, F2, F3, F4, and F5 administrations increased IL-10 secretion in a dose-dependent manner by LPS-stimulated splenocytes, however only F4 and F5 administrations at the indicated concentration of 500 μg/mL significantly ($P < 0.05$) increased IL-10 secretion. Even though the samples treated with heating, HF3, HF4, and HF5 still significantly increased IL-10 secretions (Table 2). The results from this study suggest that anti-inflammatory pigment compounds in red cabbage juice were heat stable.

3.3. Total phenolic and flavonoid contents in selected chromatographic fractions from red cabbage juice through Sephadex LH-20 column

To determine the contents of phenolics including flavonoids in red cabbage juice, gallic acid which is a major phenolic compound in red cabbage and naringenin which is a main flavonoid component in red cabbage were chosen as standards for phenolics and flavonoids, respectively. The total phenolic, and flavonoid contents, as g gallic acid, and naringenin equivalent/100 g powder, respectively, in each of the five fractions of red cabbage separated on Sephadex LH-20 column are given in Table 3. Results reveal that fractions F2, F3, and F4 among the five fractions were rich in phenolics ($5.9 \pm 0.2\%$, $4.4 \pm 0.0\%$, and $3.9 \pm 0.0\%$, respectively) and flavonoid ($1.8 \pm 0.3\%$, $1.8 \pm 0.3\%$, and $1.1 \pm 0.3\%$, respectively). However, total phenolic and flavonoid levels in chromatographic fractions were decreased as the eluent volume was increased (from F2 fraction to F5 fraction). Except the total phenolic level in the F2 fraction, heating did not significantly ($P > 0.05$) changed the levels of total phenolic and flavonoid contents.

3.4. High performance liquid chromatography (HPLC) of red cabbage juice and its chromatographic fractions through Sephadex LH-20 column

The HPLC chromatographic characteristics recorded at 530 nm for crude red cabbage juice (RC), and its chromatographic fractions (F1–F5) through Sephadex LH-20 column as well as anthocyanin standards are shown in Table 4. The distinguishable peak in the HPLC chromatograms is represented as retention time (RT). The results showed that the major pigment compounds of red cabbage juice were distributed in the fractions 2, 3, and 4 while both fractions 1 and 5 contained no significant pigment compounds. Based on RT in HPLC column, two groups of pigments, respectively, reflecting RT 3–7 min and 70–76 min were identified. The identified pigment compounds according

Table 1
Effects of red cabbage juice administrations on pro- and anti-inflammatory cytokine secretions by LPS-stimulated splenocytes

Treatments	Cytokines			
	Pro-inflammatory cytokines			Anti-inflammatory cytokine
	IL-1β (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)
LPS stimulation only	16.5 ± 5.4	1168 ± 350	357 ± 51	861 ± 180
LPS + 50μg/mL of RC	20.7 ± 11.8	1043 ± 278	271 ± 81	$1848 \pm 741^{*}$
LPS + 250μg/mL of RC	13.3 ± 6.2	822 ± 290	395 ± 151	$1496 \pm 247^{*}$
LPS + 1250μg/mL of RC	26.5 ± 15.0	$318 \pm 73^{*}$	412 ± 78	1575 ± 456

Data are shown as mean ± SD ($n = 3$). Asterisk (*) means significantly different ($P < 0.05$) from the positive control of LPS stimulation only in the same column. The IL-1β, IL-6, TNF-α, and IL-10 levels in the negative control culture were 1.5 ± 1.1, 62.9 ± 15.4, 27.1 ± 49.2, and 40.3 ± 86.8 pg/mL, respectively. The lipopolysaccharides (LPS) concentration used in this study was 10 μg /mL. The sensitivity of these ELISA kits was <15.6 pg/mL. RC: red cabbage juice.

Table 2
Effects of administrations with red cabbage juice and its chromatographic fractions through Sephadex LH-20 column on IL-6 and IL-10 secretions by LPS-stimulated splenocytes

Treatments	IL-6 secretion (pg/mL)					
	RC	F1	F2	F3	F4	F5
LPS stimulation only	1252 ± 89	1252 ± 89	1252 ± 89	1252 ± 89	1252 ± 89	1252 ± 89
LPS + samples (20 μg/mL)	1380 ± 112	1185 ± 70	1209 ± 130	1217 ± 98	1134 ± 136	1253 ± 89
LPS + samples (100 μg/mL)	1292 ± 124	1014 ± 94**	1009 ± 123**	1035 ± 124**	1076 ± 167*	1203 ± 80
LPS + samples (500 μg/mL)	985 ± 84**	676 ± 131**	980 ± 174**	784 ± 86**	1164 ± 70	1312 ± 76
	HRC	HF1	HF2	HF3	HF4	HF5
PS stimulation only	685 ± 24	685 ± 24	685 ± 24	685 ± 24	685 ± 24	567 ± 101
LPS + samples (20 μg/mL)	575 ± 135	599 ± 121	543 ± 78*	545 ± 181	504 ± 70*	527 ± 105
LPS + samples (100 μg/mL)	428 ± 195	696 ± 105	444 ± 109*	464 ± 62**	488 ± 110*	527 ± 105
LPS + samples (500 μg/mL)	588 ± 31*	792 ± 169	388 ± 129*	538 ± 233	473 ± 202	734 ± 76
	IL-10 secretion (pg/ml)					
	RC	F1	F2	F3	F4	F5
LPS stimulation only	508 ± 66	508 ± 66	508 ± 66	508 ± 66	508 ± 66	508 ± 66
LPS + samples (20 μg/mL)	503 ± 120	541 ± 202	498 ± 155	472 ± 114	533 ± 124	428 ± 337
LPS + samples (100 μg/mL)	569 ± 158	669 ± 206	500 ± 120	526 ± 147	646 ± 185	624 ± 135
LPS + samples (500 μg/mL)	641 ± 198	687 ± 227	724 ± 238	710 ± 231	850 ± 183*	1028 ± 171**
	HRC	HF1	HF2	HF3	HF4	HF5
LPS stimulation only	784 ± 132	784 ± 132	784 ± 132	784 ± 132	784 ± 132	784 ± 132
LPS + samples (20 μg/mL)	521 ± 4	823 ± 477	947 ± 279	1009 ± 19*	1146 ± 309	712 ± 551
LPS + samples (100 μg/mL)	462 ± 188	1471 ± 884	1032 ± 589	1164 ± 165*	1738 ± 398*	1316 ± 984
LPS + samples (500 μg/mL)	854 ± 346	1272 ± 390	631 ± 226	1173 ± 371	2676 ± 989*	2516 ± 868*

Data are shown as mean ± SD ($n = 3$). Asterisks (*, **) mean significantly different from the positive control (LPS stimulation only) within same column at the levels of $P < 0.05$, or $P < 0.01$, respectively. The IL-6 level in the negative control culture was 93 ± 70 pg/mL. The IL-10 levels in negative control cultures were 14 ± 30 and 34 ± 43 pg/mL, respectively. The lipopolysaccharides (LPS) concentration used in this study was 10 μg/mL. The sensitivity of these ELISA kits was <15.6 pg/mL. RC: red cabbage juice; HRC: heated RC; Fs: chromatographic fractions; HFs: heated chromatographic fractions.

to RT were peaks (1) 3.03 min, (2) 6.99 min, (3) 69.92 min, (4) 71.49 min, (5) 74.11 min, (6) 75.39 min, and (7) 76.19 min. The maximum and minimum pigment contents (area) in RC were peaks (4) 71.49 min and (5) 74.11 min, respectively. The RC, F2, F3, and F4 shared similar HPLC chromatograms (Table 4). The pigment compounds in RC through Sephadex LH-20 column were finally distributed in F2 and F3. To further identify the anthocyanin compounds in red cabbage juice, two anthocyanin standards, delphinidin (RT: 22.37 min) and malvidin (RT: 74.75 min), were subjected to the HPLC analysis. Peaks identified in RC corresponded to malvidin (peak 5), and suggested to be malvidin glycosides (peaks 3 and 4), and oenin (peak 1). However, delphinidin seemed not to be the major pigment in RC. More LC–MS–MS works should be done in order to confirm the structures of anthocyanins in RC.

3.5. The correlation among known pigment component contents in crude red cabbage juice as well as its chromatographic fractions and pro-inflammatory cytokine IL-6 secretion

The relationship among individual anthocyanin contents in red cabbage juice as well as its chromatographic fractions, and anti-inflammatory effects on IL-6 secretion

Table 3
Total phenolic and flavonoid contents in selected chromatographic fractions from red cabbage juice through Sephadex LH-20 column

Compositions (g/100 g lyophilized powder)		Chromatographic fractions				
		F1	F2	F3	F4	F5
Phenolics	Non-heated	–	5.9 ± 0.2[a]	4.4 ± 0.0	3.9 ± 0.0	0.2 ± 0.0
	Heated	–	5.1 ± 0.2[b]	4.6 ± 0.4	4.0 ± 0.0	0.2 ± 0.0
Flavonoid	Non-heated	–	1.8 ± 0.3	1.8 ± 0.3	1.1 ± 0.3	0.6 ± 0.2
	Heated	–	1.9 ± 0.0	2.1 ± 0.1	1.0 ± 0.4	0.6 ± 0.0

Data are shown as mean ± SD ($n = 3$). "–" indicates not determined. Total phenolic, and flavonoid contents are expressed of as g gallic acid, and naringenin equivalent/100 g powder, respectively. Within same composition column not sharing a letter are significant different ($P < 0.05$) according to Duncan's New Multiple Range test.

Table 4
HPLC chromatographic characteristics of red cabbage juice, and its chromatographic fractions through Sephadex LH-20 column as well as anthocyanin standards

Samples	Peak number	Retention time (min)	Peak area
Red cabbage juice	1	3.03	2,588,470
	2	6.99	874,867
	3	69.92	3,711,174
	4	71.49	4,256,400
	5	74.11	552,470
	6	75.39	773,981
	7	76.19	809,792
			13,567,154
F1	Undetectable		
F2	1	3.03	3,039,967
	2	6.61	954,143
	3	70.59	2,549,640
	4	71.97	4,180,533
	5	74.43	402,409
	6	75.55	347,179
	7	76.29	697,853
			12,171,724
F3	1	3.05	1,181,934
	2	7.04	474,239
	3	70.59	4,240,476
	4	71.92	4,294,072
	5	74.43	1,474,961
	6	75.55	1,417,178
	7	76.24	1,452,542
			14,535,402
F4	1	3.03	1,505,251
	2	6.90	516,545
	3	70.69	1,235,356
	4	71.97	1,347,587
	5	74.48	154,736
	6	75.60	124,503
	7	76.35	118,683
			5,002,661
F5	Undetectable		
Delphinidin	1	18.6	389,314
	2	22.37	14,862,480
Malvidin	1	74.75	2,318,374

were, respectively, statistically analyzed. The correlation among oenin (RT 3.03 min in HPLC) contents in red cabbage juice as well as its chromatographic fractions, and IL-6 secretions are shown as Fig. 2a. The correlation coefficient (R) amount between oenin content (area) and IL-6 secretion by LPS-stimulated splenocytes was -0.957 ($P = 0.024$). The result suggests that oenin in red cabbage juice significantly contributed to inhibit IL-6 secretion of LPS-stimulated splenocytes and exhibited anti-inflammatory potential *in vitro*. Further analysis showed that malvidin glycosides (RT at 69.92 min and RT at 71.49 min in HPLC) in red cabbage juice significantly ($P < 0.05$) decreased IL-6 secretions and demonstrated anti-inflammatory potential *in vitro* (Fig. 2b and c). Although the correlation coefficient (R) between malvidin (RT 74.11 min) content (area) and IL-6 secretion was -0.850 ($P = 0.064$), malvidin itself could not significantly inhibit IL-6 secretion (Fig. 2d). Both malvidin glycosides and oenin contents of red cabbage juice showed a significantly ($P < 0.05$) negative correlation with IL-6 secretion by LPS-stimulated splenocytes.

4. Discussion

Anthocyanins which responsible for the appealing and spectacular orange, red purple and blue colours of many fruits, vegetables, flowers, leaves, root and other plant storage organs have been applied in food systems (Giusti & Wrolstad, 2003). Besides the colour attributes, anthocyanins seem to have many physiological functions including anti-inflammatory activities (Heo & Lee, 2006). In this study red cabbage juice exhibited an anti-inflammatory effect against LPS-induced inflammation of primary splenocytes via increasing anti-inflammatory cytokine IL-10 and decreasing pro-inflammatory cytokine IL-6 secretions (Table 1). Lee et al. (2002) have reported that red cabbage extracts, by oral gavage, exhibited a neuroprotective action in brain of mice administered i.p. with *N*-methyl-D-aspartate (NMDA) (Lee et al., 2002). The present study further suggests the anti-inflammatory activity of red cabbage juice via immuno-modulating the secretions of pro- and anti-inflammatory cytokines.

To characterize pigments in plant, UV–vis molecular absorption spectrophotometry is one of the most widespread methods (Drabent, Pliszaka, & Olszewska, 1999). Deep-coloured vegetables are rich in phenolics including flavonoids and anthocyanins (Lin & Tang, 2007). Anthocyanins are also flavonoids. Although the absorption spectra among phenolics, flavonoids, and anthocyanins may overlap, the major absorption peaks among phenolics, flavonoids, and anthocyanins are characteristic. According to the absorption spectra of red cabbage juice (Fig. 1b–d), we suggested that pigments in red cabbage juice might include phenolics (absorption peak around 280 nm) (Chirinos et al., 2008), flavonoids (absorption peak around 310–330 nm) (Merken & Beecher, 2000) and anthocyanins (absorption peak around 480–630 nm) (Takeda et al., 1994). Phenols absorbs in the ultraviolet (UV) region, while flavonoids are characterized with a maximum absorption band in the 240–285 nm range due to their A-ring and with another maximum absorption band in the 300–550 nm range due to their B-ring (Merken & Beecher, 2000). Anthocyanins show absorption maxima in the 265–275 and 465–560 nm regions, respectively (Merken & Beecher, 2000). The anthocyanin which is responsible for purplish blue colour is very stable in neutral or slightly acidic aqueous solution and exhibits three characteristic bands at 534, 569, and 620 nm (Takeda et al., 1994). However, the pigment complex such as metallo-anthocyanins, flavonol copigments, glycosides, and polymerization, etc. may slightly modify the characteristics of absorption spectra (Asen, Stewart, & Norris, 1975; Merzlyak, Solovchenko, & Smagin, 2005; Takeda, Yanagisawa, Kifune, Kinoshita,

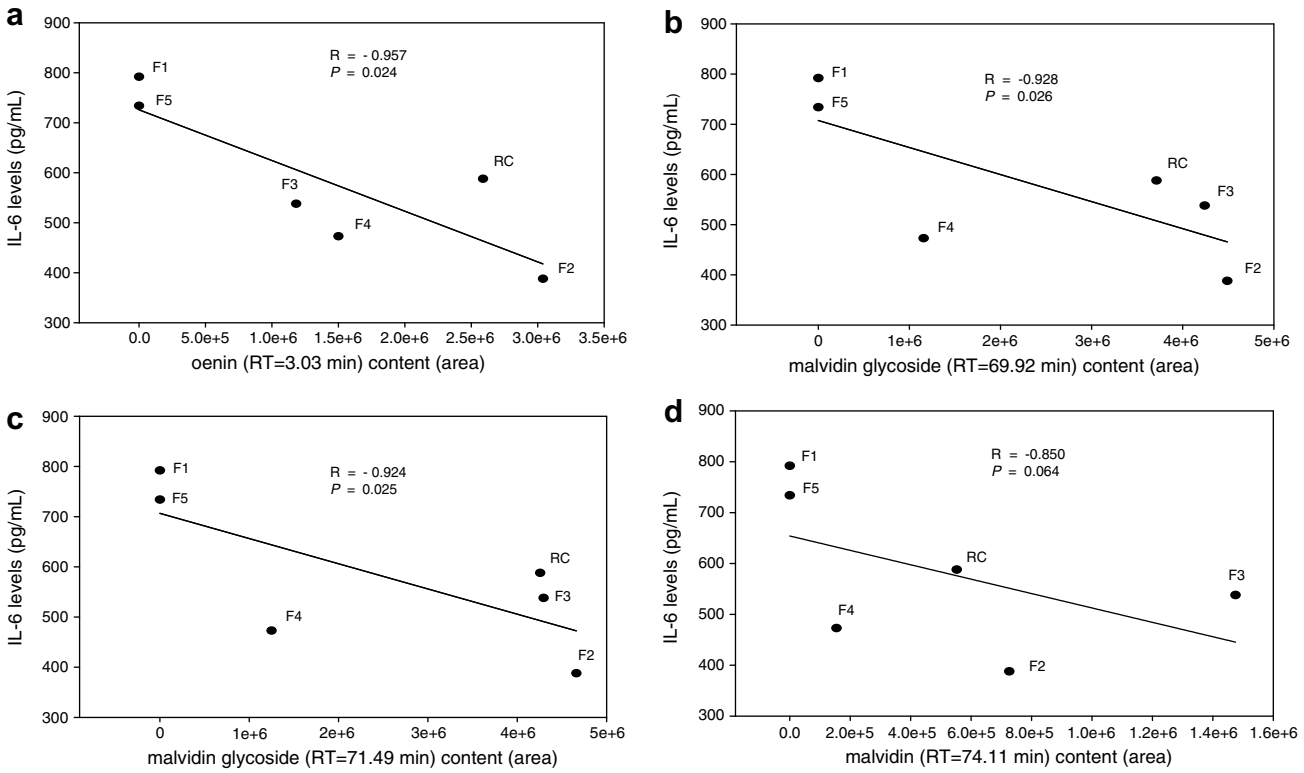

Fig. 2. The correlations among oenin (a), malvidin and its glycoside contents (b–d) in red cabbage juice as well as its chromatographic fractions through Sephadex LH-20 column and IL-6 secretions by LPS-stimulated splenocytes.

& Timberlake, 1994). Eight to 15 anthocyanins in *B. oleracea* L. have been found, which exist as 5-glucosides and 3,5-diglucosides with different anthocyanidins as chromophoric groups (Drabent et al., 1999). Although most proteins also show a maximum absorption band around 280 nm (Yu et al., 2006), the disappearance of absorption peaks in the UV–vis spectra of red cabbage juice after being treated with activated charcoal (Fig. 1b), and the heat stable property (Fig. 1b, Table 2) suggest that proteinaeous substances in red cabbage juice might not be the major bioactive constitutes against inflammation. This study suggests that phenolics and flavonoids in red cabbage juice are the major contributors for anti-inflammation (Table 3). However, the individual components remain to be further clarified.

To unravel which anthocyanins in red cabbage juice have an anti-inflammatory activity, red cabbage juice and its chromatographic fractions (F1–F5), as well as some anthocyanin standards were subjected to HPLC analysis. The HPLC chromatograms were recorded at 530 nm. Two groups of pigments in RC distributed at RT of 3–7 min and 70–76 min were found (Table 4). The malvidin (RT: 74.75 min) was found to exist in red cabbage juice. Anthocyanin glycosides have shorter retention time than that of the same anthocyanins in the same HPLC column (Kader et al., 1995). Thus, peaks identified in RC corresponded to malvidin (peak 5, RT: 74.11 min), and suggested to be malvidin glycosides (peak 3, RT: 69.92 min and peak 4, RT: 71.49 min) (Table 4). We suggest the malvidin glycosides in red cabbage juice are malvidin 5-glucoside and malvidin 3,5-diglucoside (Drabent et al., 1999). However, more evidences should be acquired. Referred to the established HPLC chromatograms of anthocyanins under the same condition (Shih, 2004), the pigment at RT 3.03 min in RC was identified to be oenin (malvidin 3-glucoside) (Berke & de Freitas, 2005), the major red pigment of the grape skins (Koeppen & Basson, 1966). This study further suggests that oenin is also rich in red cabbage juice (Table 4). The oenin (malvidin 3-glucoside) may react with catechin in the presence of vanillin to form an anthocyanin-aryl-flavanol adduct, which has a maximum absorption wavelength in the visible region (λ_{max}) of 549 nm, attributed to a purple colour (Sousa et al., 2007). This study suggested that oenin in RC may play an important physiological function.

In this study we attempted to investigate the relationship among contents of authentic anthocyanins in red cabbage juice as well as its chromatographic fractions, and anti-inflammatory effects on IL-6 secretion. The results exhibited significantly negative correlations among oenin (malvidin 3-glucoside, RT 3.03 min in HPLC) as well as malvidin glycosides (RT 69.92 min and RT 71.49 min in HPLC) contents, and IL-6 secretions (Fig. 2a–c). However, malvidin (RT 74.11 min) itself could not significantly inhibit IL-6 secretion (Fig. 2d). Hou et al. (2005) also reported that malvidin could not inhibit COX-2 expression in LPS-activated

murine macrophage RAW264, suggesting malvidin has little anti-inflammatory potential (Hou et al., 2005). The present study provides supporting evidence for the use of red cabbage juice as an anti-inflammatory agent against inflammed murine splenocytes *in vitro*. To unravel more pharmacological actions of the red cabbage juice, the bioactive compounds should be further purified and used in animal studies.

5. Conclusions

Red cabbage juice *in vitro* exhibited anti-inflammatory effects against LPS-induced inflammation of murine primary splenocytes via increasing anti-inflammatory cytokine IL-10 and decreasing pro-inflammatory cytokine IL-6 secretions. HPLC chromatograms further suggested that malvidin glycosides, including malvidin 3-glucoside (oenin), malvidin 5-glucoside and malvidin 3,5-diglucoside in red cabbage juice could be responsible for inhibition of the IL-6 secretion of LPS-stimulated splenocytes.

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References

Asen, S., Stewart, R. N., & Norris, K. H. (1975). Anthocyanin, flavonol copigments, and pH responsible for larkspur flower color. *Phytochemistry, 14*, 2677–2682.

Berke, B., & de Freitas, V. A. P. (2005). Influence of procyanidin structures on their ability to complex with oenin. *Food Chemistry, 90*, 453–460.

Chang, C. C., Yang, M. H., Wen, H. M., & Chern, J. C. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *Journal of Food and Drug Analysis, 10*, 178–182.

Chen, P.-N., Chu, S.-C., Chiou, H.-L., Kuo, W.-H., Chiang, C.-L., & Hsieh, Y.-S. (2006). Mulberry anthocyanins, cyanidin 3-rutinoside and cyanidin 3-glucoside, exhibited an inhibitory effect on the migration and invasion of a human lung cancer cell line. *Cancer Letters, 235*, 248–259.

Chigurupati, N., Saiki, L., Gayser, C., & Dash, A. K. (2002). Evaluation of red cabbage dye as a potential natural color for pharmaceutical use. *International Journal of Pharmaceutics, 241*, 293–299.

Chirinos, R., Campos, D., Costa, N., Arbizu, C., Pedreschi, R., & Larondelle, Y. (2008). Phenolic profiles of andean mashua (*Tropaelum tuberosum* Ruiz & Pavon) tubers: Identification by HPLC-DAD and evaluation of their antioxidant activity. *Food Chemistry*, *106*, 1285–1298.

Drabent, R., Pliszaka, B., & Olszewska, T. (1999). Fluorescence properties of plant anthocyanin pigments. I. Fluorescence of anthocyanins in *Brassica oleracea* L. extracts. *Journal of Photochemistry and Photobiology B: Biology, 50*, 53–58.

Frostegard, J., Ulfgren, A.-K., Nyberg, P., Hedin, U., Swedenborg, J., Andersson, U., et al. (1999). Cytokine expression in advanced human atherosclerotic plaques: Dominance of pro-inflammatory (Th1) and macrophage-stimulating cytokines. *Atherosclerosis, 145*, 33–43.

Gil, A. (2002). Polyunsaturated fatty acids and inflammatory diseases. *Biomedicine & Pharmacotherapy, 56*, 388–396.

Giusti, M. M., & Wrolstad, R. (2003). Acylated anthocyanins from edible sources and their applications in food systems. *Biochemical Engineering Journal, 14*, 217–225.

Hanada, T., & Yoshimura, A. (2002). Regulation of cytokine signaling and inflammation. *Cytokine & Growth Factor Reviews, 13*, 413–421.

Heimler, D., Vignolini, P., Dini, M. G., Vincieri, F. F., & Romani, A. (2006). Antiradical activity and polyphenol composition of local *Brassicaceae* edible varieties. *Food Chemistry, 99*, 464–469.

Heo, H. J., & Lee, C. Y. (2006). Phenolic phytochemicals in cabbage inhibit amyloid β protein-induced neurotoxicity. *LWT—Food Science and Technology, 39*, 330–336.

Hou, D.-X., Yanagita, T., Uto, T., Masuzaki, S., & Fujii, M. (2005). Anthocyanidins inhibit cyclooxygenase-2 expression in LPS-evoked macrophages: Structure–activity relationship and molecular mechanisms involved. *Biochemical Pharmacology, 70*, 417–425.

Iqbal, M. J., & Ashiq, M. N. (2007). Adsorption of dyes from aqueous solutions on activated charcoal. *Journal of Hazardous Materials B, 139*, 57–66.

Jara, L. J., Medina, G., Vera-Lastra, O., & Amigo, M.-C. (2006). Accelerated atherosclerosis, immune response and autoimmune rheumatic diseases. *Autoimmunity Reviews, 5*, 195–201.

Kader, F., Rovel, B., Girardin, M., & Metche, M. (1995). Fractionation and identification of the phenolic compounds of high blueberries. *Food Chemistry, 55*, 35–40.

Karin, M., Lawrence, T., & Nizet, V. (2006). Innate immunity gone awry: Linking microbial infections to chronic inflammation and cancer. *Cell, 124*, 823–835.

Kim, K.-M., Kwon, Y.-G., Chung, H.-T., Yun, Y.-G., Pae, H.-O., Han, J.-A., et al. (2003). Methanol extract of *Cordyceps pruinosa* inhibits in vitro and in vivo inflammatory mediators by suppressing NF-κB activation. *Toxicology and Applied Pharmacology, 190*, 1–8.

Koeppen, B. H., & Basson, D. S. (1966). The anthocyanin pigments of barlinka grapes. *Phytochemistry, 5*, 183–187.

Lee, K.-J., Sok, D.-E., Kim, Y.-B., & Kim, M. R. (2002). Protective effect of vegetable extracts on stress in brain of mice administered with NMDA. *Food Research International, 35*, 55–63.

Li, J.-J., Guo, Y.-L., & Yang, Y.-J. (2005). Enhancing anti-inflammatory cytokine IL-10 may be beneficial for acute coronary syndrome. *Medical Hypotheses, 65*, 103–106.

Lin, J.-Y., Lai, Y.-S., Liu, C.-J., & Wu, A.-R. (2007). Effects of lotus plumule supplementation before and following systemic administration of lipopolysaccharide on the splenocyte responses of BALB/c mice. *Food and Chemical Toxicology, 45*, 486–493.

Lin, J.-Y., & Tang, C.-Y. (2007). Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. *Food Chemistry, 101*, 140–147.

Lin, J.-Y., & Tang, C.-Y. (2008a). Total phenolic contents in selected fruit and vegetable juices exhibit a positive correlation with interferon-γ, interleukin-5, and interleukin-2 secretions using primary mouse splenocytes. *Journal of Food Composition and Analysis, 21*, 45–53.

Lin, J.-Y., & Tang, C.-Y. (2008b). Strawberry, loquat, mulberry, and bitter melon juices exhibit prophylactic effects on LPS-induced inflammation using murine peritoneal macrophages. *Food Chemistry, 107*, 1587–1596.

Manach, C., Scalbert, A., Morand, C., Remesy, C., & Jimenez, L. (2004). Polyphenols: Food sources and bioavailability. *American Journal of Clinical Nutrition, 79*, 727–747.

McDougall, G. J., Fyffe, S., Dobson, P., & Stewart, D. (2007). Anthocyanins from red cabbage—Stability to simulated gastrointestinal digestion. *Phytochemistry, 68*, 1285–1294.

Meda, A., Lamien, C. E., Romito, M., Millogo, J., & Nacoulma, O. G. (2005). Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chemistry, 91*, 571–577.

Merken, H. M., & Beecher, G. R. (2000). Measurement of food flavonoids by high-performance liquid chromatography: A review. *Journal of Agricultural and Food Chemistry, 48*, 577–599.

Merzlyak, M. N., Solovchenko, A. E., & Smagin, A. I. (2005). Apple flavonols during fruit adaptation to solar radiation: Spectral features and technique for non-destructive assessment. *Journal of Plant Physiology, 162*, 151–160.

Prior, R. L. (2003). Fruits and vegetables in the prevention of cellular oxidative damage. *American Journal of Clinical Nutrition, 78*, 570S–578S.

Roybal, J. E., Pfenning, A. P., Turnipseed, S. B., & Gonzales, S. A. (2003). Application of size-exclusion chromatography to the analysis of shrimp for sulfonamide residues. *Analytica Chimica Acta, 143*, 147–152.

Saleem, A., Husheem, M., Harkonen, P., & Pihlaja, K. (2002). Inhibition of cancer cell growth by crude extract and the phenolics of *Terminalia chebula* retz. fruit. *Journal of Ethnopharmacology, 81*, 327–336.

Saleh, N. A. M., Poulton, J. E., & Grisebach, H. (1976). UDP-glucose: Cyanidin 3-*O*-glucosyltransferase from red cabbage seedlings. *Phytochemistry, 15*, 1865–1868.

Sarkar, D., & Fisher, P. B. (2006). Molecular mechanisms of aging-associated inflammation. *Cancer Letters, 236*, 13–23.

Shih, P.-H. (2004). Molecular mechanisms of anthocyanin-induced apoptosis in human gastric adenocarcinoma cells. Master Thesis of National Chung Hsing University, Taichung, Taiwan.

Singh, J., Upadhyay, A. K., Bahadur, A., Singh, B., Singh, K. P., & Rai, M. (2006). Antioxidant phytochemicals in cabbage (*Brassica oleracea* L. var. capitata). *Scientia Horticulturae, 108*, 233–237.

Sousa, C., Mateus, N., Silva, A. M. S., Gonzalez-Paramas, A. M., Santos-Buelga, C., & de Freitas, V. (2007). Structural and chromatic characterization of a new malvidin 3-glucoside-vanillyl-catechin pigment. *Food Chemistry, 102*, 1344–1351.

Takeda, K., Sato, S., Kobayashi, H., Kanaitsuka, Y., Ueno, M., Kinoshita, T., et al. (1994). The anthocyanin responsible for purlish blue flower colour of *Aconitum chinense*. *Phytochemistry, 36*, 613–616.

Takeda, K. K., Yanagisawa, M., Kifune, T., Kinoshita, T., & Timberlake, C. F. (1994). A blue pigment complex in flowers of *Salvia patens*. *Phytochemistry, 35*, 1167–1169.

Tanchev, S. S., & Timberlake, C. F. (1969). The anthocyanins of red cabbage (*Brassica oleracea*). *Phytochemistry, 8*, 1825–1827.

Yu, Y., Hu, J., Miyaguchi, Y., Bai, X., Du, Y., & Lin, B. (2006). Isolation and characterization of angiotensin I-converting enzyme inhibitory peptides derived from porcine hemoglobin. *Peptides, 27*, 2950–2956.

Zhang, Y., Vareed, S. K., & Nair, M. G. (2005). Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. *Life Sciences, 76*, 1465–1472.

Zin, Z. M., Hamid, A. A., Osman, A., & Saari, N. (2006). Antioxidative activities of chromatographic fractions obtained from root, fruit and leaf of Mengkudu (*Morinda citrifolia* L.). *Food Chemistry, 94*, 169–178.