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In vitro evaluation of red and green lettuce (*Lactuca sativa*) for functional food properties

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ABSTRACT

Lettuce (*Lactuca sativa*) is an important leafy vegetable consumed fresh or in salad mixes. We have compared the functional food properties of selected commercial red and green lettuce varieties grown under field conditions. Both lettuce cultivars were extracted with water at biological (38 °C) and room temperatures (22 °C) at pH 2. The residues from each extraction were further extracted, sequentially with methanol and ethyl acetate. The extracts were evaluated for their *in vitro* lipid peroxidation (LPO) and cyclooxygenase enzyme (COX) inhibitory activities. Amongst the extracts tested, all three extracts of red lettuce showed higher LPO and COX-1 and -2 enzyme inhibitory activities than did the green lettuce extracts. Red lettuce contained a single anthocyanin, cyanidin-3-*O*-(6″-malonyl-β-glucopyranoside) (**1**), which immediately converted to cyanidin-3-*O*-(6″-malonyl-β-glucopyranoside methyl ester) (**2**) and cyanidin-3-*O*-β-glucopyranoside (**3**) under laboratory conditions. Anthocyanins **1** and **2** inhibited LPO by 88% and 91.5%, respectively, at 0.25 μM concentration. Also, they inhibited COX-2 enzyme by 78.9% and 84.3% and COX-1 by 64% and 65.8%, respectively, at 5 μM. The chicoric acid (**4**), amongst other phenolics, such as quercetin glucoside, ferulic and caffeic acids, isolated from both green and red lettuce, showed 85.6%, 45.6% and 94% of LPO, COX-1 and -2 enzyme inhibitions at 50 μM, respectively. This is the first report of the LPO, COX-1 and -2 enzyme inhibitory activities of compounds **1**, **2** and **4**. The variation of phenolics in the red and green lettuces, and specifically the lack of anthocyanins in green lettuce, might account for the higher biological activity obtained with the red variety in our study.

1. Introduction

Numerous studies have shown a correlation between the consumption of fresh fruits and vegetables and their health benefits. Epidemiological studies have further demonstrated the relationship between dietary habits and disease risk and established that food has a direct impact on health. Lettuce, *Lactuca sativa*, is an important dietary leafy vegetable that is primarily consumed fresh or in salad mixes due to its perception as being amongst healthier foods (Dupont, Mondi, Willamson, & Price, 2000). A number of lettuce varieties have been investigated recently and reported to contain phenolic compounds with antioxidant activities (Llorach, Martinez-Sanchez, Tomas-Barberan, Gil, & Ferrers, 2008; Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995). The health benefits of lettuce have also been attributed to the presence of Vitamin C, phenolic compounds and fibre content (Nicolle & Cardinault et al., 2004; Nicolle & Carnat et al., 2004. In folk medicine, lettuce seeds are used in the treatment of asthma, cough and as an analgesic.

In 2006, over 4,338,000 metric tons of lettuce plants were harvested from over 306,600 acres with a total value of over $2 billion. Iceberg, romaine and leaf lettuces represented 61%, 18% and 21% of the total national production (by weight), respectively. Despite a relatively small proportion of the total production (21%), leaf lettuce, including red and green types, continues to have the highest value. In 2006, leaf lettuce was valued at $8,451/acre compared to $5,596/acre and $7,016/acre for iceberg and romaine types, respectively. Iceberg lettuce is so far the most common lettuce used (especially in fast food restaurants). The antioxidant phenolics in lettuce vary amongst varieties due to growing practices, processing and storage conditions (Baur, Klaiber, Koblo, & Carle, 2004). Currently, red lettuce is popular in salad mixes due to its anthocyanin content that contributes to the higher value it fetches compared to the green lettuce (Gazula, Kleinhenz, Scheerens, & Ling, 2007). The increased demand of fresh vegetables associated with health benefits has led to an increase in the quality, quantity and variety of produce available to the consumer. Various approaches, involving

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environmental, cultural and management practices, have been used to enhance the quality of lettuce, specifically in the areas of phytochemical contents and health-promoting attributes (Kleinhenz, French, Gazula, & Scheerens, 2003).

Anthocyanins are water-soluble phenolic glycosides that colour fruits, flowers, vegetables and cereals. Apart from imparting colour to plants, anthocyanins exhibit an array of health-promoting benefits. We have reported that anthocyanins isolated from various plants inhibited lipid peroxidation (LPO) and cyclooxygenase (COX) enzymes (Mulabagal, Van Nocker, DeWitt, & Nair, 2007; Seeram, Cichewez, Chandra, & Nair, 2003; Tall, Seeram, Zhao, Nair, & Meyer, 2004; Wang, Nair, & Strasburg, 1999). The ability of anthocyanidins to inhibit LPO and COX enzymes has also been reported (Seeram & Nair, 2002). Both anthocyanins and anthocyanidins stimulated insulin release by rodent pancreatic β-cells (INS-1 832/13) *in vitro* (Jayaprakasam, Vareed, Olson, & Nair, 2005). Also, a purified anthocyanin mixture from *Cornus mas* fruits has demonstrated an ability to ameliorate obesity and insulin resistance in C57BL/6 mice fed a high-fat diet (Jayaprakasam, Olson, Schutzki, Tai, & Nair, 2006). In another *in vivo* study, anthocyanins up-regulated the adipocyte-specific gene and genes involved in lipid metabolism (Tsuda, Ueno, Kojo, Yoshikawa, & Osawa, 2005; Tsuda et al., 2004).

Although red lettuce costs more than green lettuce, it is becoming very popular amongst consumers. This is probably due to its red colour and its association with better health as in the case of red fruits and berries. Therefore, in this study, we have compared both green and red lettuce, using *in vitro* bioassays and chemical composition studies and evaluated their functional food advantages.

2. Materials and methods

2.1. Materials

Seeds of green (Var. North Star) and red lettuce (Var. Cherokee) were purchased from Siegers Seeds Company (Holland, MI). The COX-1 enzyme was prepared from ram seminal vesicles purchased from Oxford Biomedical research, Inc. (Oxford, MI). The COX-2 enzyme was prepared from prostaglandin endoperoxide-H synthase-2 (PGHS-2)-cloned insect cell lysate. Solvents used for isolation and purification were of ACS grade and purchased from Sigma–Aldrich Chemical Co., Inc. (St. Luis, MO). Positive controls, t-butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), used in the anti-oxidant assay, were purchased from Sigma Chemical Company.

2.2. Equipment

Samples were homogenised using a Kinematica CH-6010 (Roxdale, ON, Canada) homogenizer and centrifuged (model RC5C, Sorvall Instruments, Hoffman Estates, IL) at 10,000*g* for 20 min at 4 °C. The NMR (^{1}H & ^{13}C) experiments were recorded on Varian INOVA (300 MHz) and VRX (500 MHz) instruments. The chemical shifts were measured in DMSO-*d6* and CD_3OD/DCl solutions and are expressed in δ (ppm). Fractionation of anthocyanin was carried out on a XAD-2 column (500 g, Amberlite resin, mesh size 20–50; Sigma Chemical Co., St. Louis, MO) and purified on a C-18 MPLC column (350 × 40 mm). Anthocyanin detection was carried out with a Waters 2010 HPLC system (Waters Corp.) equipped with Empower Software, Shodex Degasser, Auto sampler (Waters 717) and a Photodiode Array Detector (Waters 996). HPLC analysis was carried out by using a Capcell Pak column (DyChrom, Santa Clara, CA) C-18 column (150 × 4.6 mm i.d.; 5 μm particle size). Preparative HPLC purification of anthocyanin was carried out by using a Capcell Pak (DyChrom, Santa Clara, CA) C-18 column (250 × 4.6 mm i.d.; 5 μm particle size).

2.3. Plant material

Lettuce transplants were produced in the greenhouse using 72-cell flats filled with commercial greenhouse potting mix. At the two-leaf stage, the seedlings were transplanted to the field. Field experiments were conducted at Michigan State University Horticulture Teaching and Research Center. The lettuce seedlings were transplanted on raised beds covered with black plastic mulch and drip-irrigated using two staggered rows per bed. Spacing was 30 cm between the rows and 30 cm between plants inside each row. The plants were grown in the absence of any pesticide.

2.4. LC/MS analysis

Samples were analysed on a Surveyor HPLC system equipped with a diode array absorbance detector (DAD), measuring at 520 nm, and an autosampler cooled to 4 °C (Thermo Finnigan, San Jose, USA). An Agilent Zorbax SB C-18 column, 150 × 2.1 mm, i.d.; 5 μm particle size (Agilent, USA), was used and solvent elution consisted of a gradient system over 50 min of methanol (1% acetic acid) and H_2O (1% acetic acid) at a flow rate of 0.19 ml/min. The linear gradient system started from 5% methanol (1% acetic acid) and 95% of H_2O (1% acetic acid) to 95% methanol (1% acetic acid) and 5% of H_2O (1%) at 50 min. The column was maintained at 25 °C. After passing through the flow cell of the DAD, eluate was directed to a LCQ Advantage ion trap mass spectrometer fitted with an Eelectrospray Interface (ESI). Analyses utilised the positive ion mode (*m/z* $M + H^+$) for detection of anthocyanins. Preliminary analyses were carried out using full scan, data-dependent MS/MS scanning from *m/z* 250 to 1000. The capillary temperature was set at 275 °C and the sheath and auxiliary gas at 45 and 0 units/min, respectively. The source voltage was 4 kV. MS/MS and fragmentation were carried out with 50% energy.

2.5. Extraction of lettuce

Fresh leaves of red lettuce (1.2 kg) were blended and extracted at 22 °C with acidic water (0.1% HCl, 1 l 3×) and centrifuged. Supernatants were lyophilised to get water extract (19.1 g). The residue was further extracted with methanol (500 ml 3×), followed by ethyl acetate (500 ml 3×) and the organic extracts evaporated to dryness under reduced pressure. The yields of methanol and ethyl acetate extracts were 7 and 0.2 g, respectively. Similarly, green lettuce (300 g) leaves were blended and extracted with acidic water (200 ml 3×) and the water-soluble portions were centrifuged and lyophilised (3.4 g). Residue was extracted further with methanol (100 ml 3×), followed by ethyl acetate (100 ml 3×) and the resulting extracts evaporated under vacuum to afford 0.3 and 0.4 g of dried extracts, respectively.

To mimic *in vivo* conditions, another extraction was carried out at 38 °C and pH = 2. Red lettuce leaves (350 g) were blended with acid water (0.1% HCl, 300 ml 3×) and allowed to stand at 38 °C and pH = 2 for 4 h. The mixture was then centrifuged and the supernatant lyophilised to yield a powder (14.7 g). The residue was then extracted sequentially with methanol (200 ml 3×) and ethyl acetate (200 ml 3×). The yields of methanol and ethyl acetate extracts from this procedure were 4.9 and 0.8 g, respectively. The green lettuce leaves (200 g) were also extracted in a similar manner (75 ml 3×), followed by methanol (75 ml 3×) and ethyl acetate (75 ml 3×) to afford 3.4, 0.3 and 0.4 g of dried extracts, respectively.

2.6. Purification of anthocyanin from red lettuce

The crude extract (14 g) from red lettuce was dissolved in 200 ml of water and fractionated by an XAD-2 column (35 × 6 cm) according to the published procedure (Nair, 2002). The resin with adsorbed anthocyanins was then washed with water (2 × 1 l). The water fraction was lyophilised (4.9 g). The adsorbed anthocyanin was eluted with acidic methanol (1% HCl, 2 × 1 l), concentrated at reduced pressure (35 °C) and lyophilised to yield a dark red residue (8.5 g). An aliquot of this residue (3 g) was purified further by a C-18 MPLC column (350 × 40 mm) using water:methanol (1% HCl) as the mobile phase under gradient conditions, starting with 80% H_2O. Five fractions, I (H_2O:MeOH, 80:20, 250 ml); II (H_2O:MeOH, 70:30, 200 ml); III (H_2O:MeOH, 60:40, 200 ml); IV (H_2O:MeOH, 60:40, 200 ml) and V (H_2O:MeOH, 50:50, 500 ml) were obtained. All fractions were evaporated under vacuum and analysed by HPLC for purity and anthocyanin content. Based on HPLC, Fraction II contained pure cyanidin-3-*O*-β-glucopyranoside (**3**, 12 mg) as confirmed by NMR studies (Vareed, Reddy, Schutzki, & Nair, 2006).

Fraction IV (75 mg) showed two peaks by HPLC and was further purified by preparative HPLC using Capcell Pak (4.6 × 250 mm, 5 μm). The solvent system consisted of solvent A (water-trifluoroacetic acid (TFA) 99.99:0.1 v/v) and B (water–acetonitrile–acetic acid–TFA, 50.4%, 48.5%, 1%, 0.1%, v/v/v). The linear gradient began at 80% A and 20% B, was allowed to reach 40% A and 60% B in 26 min, and then reverted back to the initial condition of 80% A and 20% B in 30 min, where it remained for 20 min. The flow rate was 1 ml/min. The peaks detected at 520 nm (Fig. 3b) were collected, evaporated to dryness and characterised by NMR spectral methods as cyanidin-3-*O*-β-glucopyranoside (**3**, 6 mg), cyanidin-3-*O*-(6″-malonyl-β-glucopyranoside (**1**, 28 mg) and the methyl ester of cyanidin-3-*O*-(6″-malonyl-β-glucopyranoside (**2**, 15 mg). It is important to note that anthocyanins **2** and **3** are conversion products of anthocyanin **1** (Fig. 3a) produced during purification in the presence of acidic methanol. The structures of anthocyanins **1, 2** and **3** were further confirmed by comparison of NMR and mass spectral data with those of published data (Andersen & Fossen, 1995; Vareed et al., 2006).

2.7. Isolation of bioactive compounds from red and green lettuce

The MeOH extract (3.6 g) of red lettuce prepared at 38 °C was subjected to a silica gel MPLC column (350 × 40 mm) and eluted with $CHCl_3$ and mixtures of $CHCl_3$ and MeOH with increasing polarity. The fractions were analysed by TLC and similar fractions were combined to give fractions A (0.28 g, $CHCl_3$:MeOH; 9:1), B (0.75 g, $CHCl_3$:MeOH; 8:2), C (1.3 g, $CHCl_3$:MeOH; 7:3) and D (0.9 g, $CHCl_3$:MeOH; 1:1). Fraction A showed a single spot on TLC, using hexane and EtOAc (8:2) as mobile phase, and was characterised as linoleic acid (15 mg). Purification of Fraction B by silica gel TLC using hexane and EtOAc mixtures as the mobile phase gave a yellow solid, quercetin (22 mg). The compounds yielded from Fraction C by silica gel MPLC purification, followed by crystallization, were ferulic (7 mg) and caffeic acids (11 mg). The purification of fraction D by silica gel TLC gave a yellow solid, chicoric acid (**4**, 6 mg). The structures of all compounds were confirmed by comparison of NMR spectral data with those of published data (Bilia, Ciampi, Mendez, & Morelli, 1996; Dutta, Mazumdar, Mishra, Dastidar, & Park, 2007; Maeda et al., 2006; Zhang, Mills, & Nair, 2003).

Methanol extract of green lettuce (300 mg), prepared at 38 °C, was also purified by silica gel MPLC, as in the case of red lettuce extract. Fractions were collected in 5 ml aliquots and analysed by TLC. Fractions that showed identical TLC profiles were combined to obtain fractions I (78 mg, chloroform:methanol, 9:1), II (40 mg, chloroform:methanol, 8:2), III (80 mg, chloroform:methanol, 6:4) and IV, (90 mg, chloroform:methanol, 1:1). Fraction I contained primarily chlorophylls and was kept aside. Further purification of fractions II–IV by silica gel MPLC and preparative TLC resulted in the isolation of linoleic acid (12 mg), quercetin glucoside (20 mg), and chicoric acid (**4**, 15 mg), respectively. Structures of the isolates were confirmed by comparison of NMR and mass spectral data with those of published data (Bilia et al., 1996; Dutta et al., 2007; Maeda et al., 2006; Zhang et al., 2003).

2.8. Lipid peroxidation inhibitory assay

Lettuce extracts, pure anthocyanins and compounds were tested *in vitro* for their abilities to inhibit the oxidation of large unilamellar vesicles (LUV's), according to published procedure (Arora & Strasburg, 1997). Peroxidation was initiated by addition of 20 μl of $FeCl_2$–$4H_2O$ (0.5 mM) for positive controls and test samples. Fluorescence was measured at 384 nm and the decrease of relative fluorescence intensity with time indicated the rate of peroxidation. Extracts were assayed at 100 ppm, anthocyanins at 0.25 μM and compound **4** at 25 μM concentrations. Positive controls t-butyl hydroquinone (TBHQ), butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), used in the anti-oxidant assay, were tested at 10 μM. Water extracts and pure anthocyanins were assayed as solutions prepared in water. Compound **4** and methanolic extracts were assayed as solutions prepared in DMSO.

2.9. Cyclooxygenase enzyme inhibitory assay

All the lettuce extracts, anthocyanins and other phenolics were tested for their COX-1, and COX-2 enzyme inhibitory assay accord-

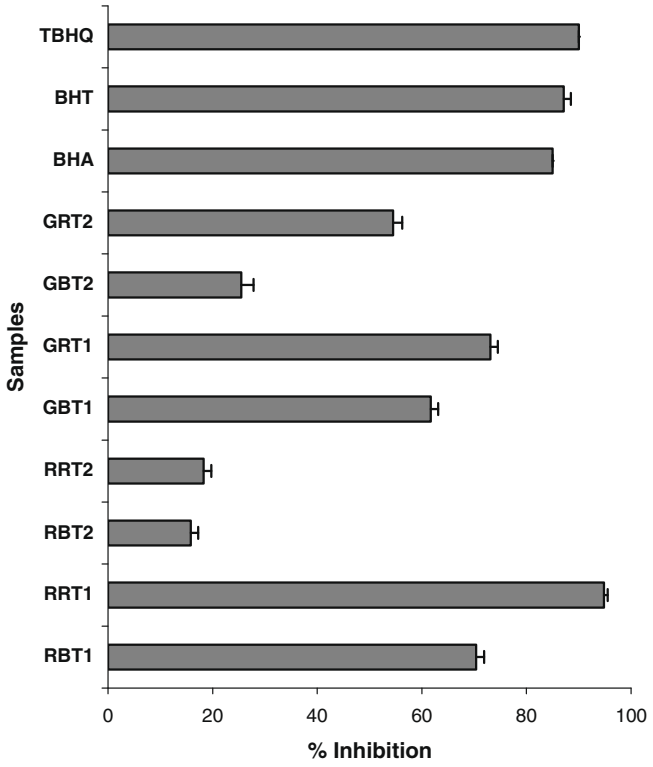

Fig. 1. Lipid peroxidation inhibitory activities of green and red lettuce extracts tested at 100 ppm (RBT1, RRT1: red lettuce water-extracts at 38 °C and 22 °C; RBT2, RRT2: red lettuce methanolic extracts of the residue from water extraction at 38 °C and 22 °C; GBT1, GRT1: green lettuce water-extracts at 38 °C and 22 °C; GBT2, GRT2: green lettuce methanolic extracts of the residue from water extraction at 38 °C and 22 °C). Positive controls BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) and TBHQ (t-butyl hydroquinone) were tested at 10 μM in DMSO. Vertical bars represent the averages of two experiments ± SD.

ing to the previously published procedure from our laboratory (Vareed et al., 2006). COX-1 enzyme was prepared from ram seminal vesicles whereas; COX-2 enzyme was prepared from insect cell lysate. The rate of oxygen consumption during the initial phase of the enzyme-mediated reaction, with arachidonic acid as substrate, was measured using a Model 5300 biological oxygen monitor (Yellow Spring Instruments Inc., Yellow Spring, OH). Aspirin (60 μM), Celebrex (26 nM), Vioxx (32 nM) and Arcoxia (120 nM) were used as positive controls. Extracts were assayed at 100 ppm, anthocyanins at 5 μM and compound **4** at 50 μM concentrations. Water extracts and pure anthocyanins were assayed as solutions prepared in water. Compound **4** and methanolic extracts were assayed as solutions prepared in DMSO.

3. Results and discussion

Although the production of red lettuce is increasing, consumers still pay a higher price for red lettuce than for green lettuce. This is probably due to the health benefits attributed to the red colour, such as by anthocyanins, in red lettuce. This assumption and price difference prompted us to compare the functional food qualities, using *in vitro* bioassays, of commercial red and green lettuce grown under field conditions.

For comparison, green and red lettuces were extracted sequentially with water and methanol under laboratory conditions (22 °C). The resulting extracts were then assayed for lipid peroxidation (LPO) and cyclooxygenase enzyme (COX) inhibitory activities at 100 ppm. Amongst the extracts tested, the water extract of red lettuce prepared at 22 and 38 °C inhibited LPO by 94.8% and 70.4%, respectively, (Fig. 1). Similarly, water extracts of green lettuce prepared at 22 and 38 °C gave 73.1% and 61.7% of LPO inhibition, respectively, at 100 ppm (Fig. 1). The COX enzyme inhibitory assays for red and green lettuce extracts were performed by using COX-1, and -2 isozymes. The water and methanol extracts of red lettuce, prepared at 22 and 38 °C, inhibited COX-2 enzyme by 89.7%, 84.9% and 87.8%, 72.2%, respectively, (Fig. 2). The water

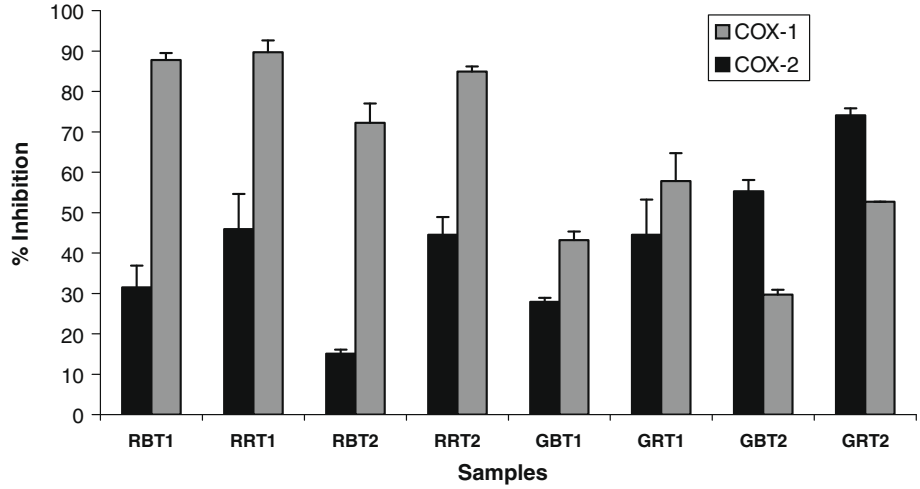

Fig. 2. *In vitro* COX-1 and COX-2 enzyme inhibitory activities of red and green lettuce extracts at 38 °C and 22 °C (RBT1, RRT1: red lettuce water-extracts at 38 °C and 22 °C; RBT2, RRT2: red lettuce methanolic extracts of the residue from water extraction at 38 °C and 22 °C; GBT1, GRT1: green lettuce water-extracts at 38 °C and 22 °C; GBT2, GRT2: green lettuce methanolic extracts of the residue after water extraction at 38 °C and 22 °C). The extracts were tested at 100 ppm. Vertical bars represent the averages of two experiments ± SD.

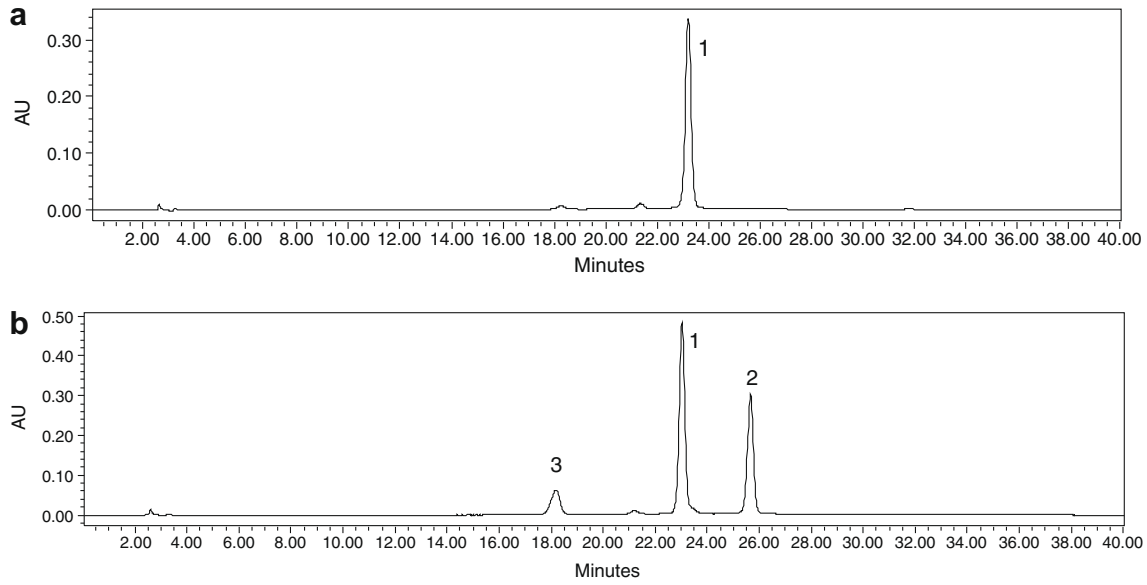

Fig. 3. (a) HPLC profile of anthocyanin in red lettuce, variety "Cherokee". Peak 1. (cyanidin 3-*O*-(6″-malonyl-β-glucopyranoside) and (b) HPLC profile of anthocyanins in the extract of red lettuce stored at room temperature for 1 h. Peak 1. (cyanidin 3-*O*-(6″-malonyl-β-D-glucopyranoside); Peak 2. (methyl ester of cyanidin-3-*O*-(6″-malonyl-β-D-glucopyranoside) and Peak 3. (cyanidin-3-*O*-β-D-glucopyranoside).

extracts of red lettuce, yielded at 22 and 38 °C, were similar in their COX-2 enzyme inhibition at 100 ppm test concentration. Water and methanol extracts of green lettuce prepared at 22 °C inhibited COX-2 enzyme by 57.8% and 52.7%, respectively, at 100 ppm (Fig. 2). Interestingly, the methanol extract of green lettuce showed 74.1% of inhibition against COX-1 enzyme (Fig. 2). Although the activity seems to be higher at 100 ppm for extracts prepared at 22 °C, the percentage weight of extracts obtained at 38 °C treatment was 3-fold higher than that of extracts at 22 °C. Therefore, the overall biological activity of fresh lettuce on a per gramme basis is higher at *in vivo* temperature than under laboratory conditions.

The primary difference between red and green lettuce is in their anthocyanin contents. Since the water extract of the red lettuce showed higher biological activity and contained anthocyanin, we have characterised the anthocyanin in the red lettuce variety studied. The HPLC profile of the extract showed only one anthocyanin, when analysed immediately after extraction, and it was characterised as cyanidin-3-*O*-(6″-malonyl-β-glucopyranoside (**1**) (Fig. 3a). However, purification or storage of the extract results in the methylation or loss of the malonic acid moiety to yield anthocyanins **2** and **3** (Figs. 3b and 4). Anthocyanins were purified using XAD-2 resin, C-18 MPLC followed by HPLC. During this process, anthocyanin **1** was converted to anthocyanin **2** (cyanidin-3-*O*-(6″-malonyl-β-glucopyranoside methyl ester) and then finally to **3** (cyanidin-3-*O*-β-glucopyranoside) (Figs. 3b and 4). The structures of anthocyanin **1** and its conversion products were elucidated by NMR and mass spectral analysis. The data were in agreement with the published spectral data of anthocyanins **1** and **2** (Andersen & Fossen, 1995). The isolation and characterisation of the acylated anthocyanins **1** and **2** from commercial red lettuce variety "Cherokee" are here reported for the first time.

The reported isolation of several anthocyanins from fruits with LPO and COX enzyme inhibitory activities (Mulabagal et al., 2007; Seeram et al., 2003; Tall et al., 2004; Wang et al., 1999), prompted us to evaluate similar biological activities of anthocyanins **1** and **2** isolated from red lettuce. Based on our experience with cyanidin-3-*O*-glucoside (**3**) and related anthocyanins, we tested **1** and **2** for LPO and COX enzyme inhibitory activities. The biological activities observed were similar to all three anthocyanins (Figs. 5 and 6). The positive controls used in the LPO assay were BHA, BHQ and TBHQ and were tested at 10 μM concentration. Similarly, anti-inflammatory drugs, Arcoxia (128 nM), Aspirin (60 μM), Celebrex (26 nM) and Vioxx (32 nM) were used as positive controls in the COX assay. Anthocyanins **1** and **2** inhibited COX-2 enzyme by 78.9% and 84.3%, respectively, when tested at 5 μM (Fig. 6). Both anthocyanins inhibited COX-1 enzyme by 64% and 65.8%, respectively, when tested at the same concentration (Fig. 6). Excellent LPO and COX inhibitory activities of anthocyanin **3** have recently been reported from our laboratory and hence were not repeated in this study (Mulabagal et al., 2007). This is the first report of the LPO, COX-1 and -2 enzyme inhibitory activities of anthocyanins **1** and **2**. Also, the activity of anthocyanins in red lettuce accounts for the higher activity observed for red lettuce than for green lettuce.

We have also analysed the methanolic extracts of red and green lettuce for bioactive principles. The red lettuce extract was fractionated by a silica gel MPLC column, using chloroform and mixtures of chloroform and methanol with increasing polarities. Low polar fractions yielded a fatty acid, linoleic acid, and a flavanone, quercetin. Medium and high polar fractions were purified to yield phenolic acids, caffeic, ferulic and chicoric (**4**) acids. The structures of these compounds were determined by comparison of NMR and mass spectral data with those of the published spectral data (Bilia et al., 1996; Dutta et al., 2007; Maeda et al., 2006; Zhang et al., 2003). This is the first report on the isolation of phenolic acids from red lettuce variety 'Cherokee'.

The purification of the methanolic extract of green lettuce resulted in the isolation of linoleic acid, quercitin glucoside, and chicoric acid (**4**). The majority of the extract contained chlorophyll, along with these compounds in minor quantities. Also, linoleic acid is considered as an essential dietary ingredient, with antioxidant

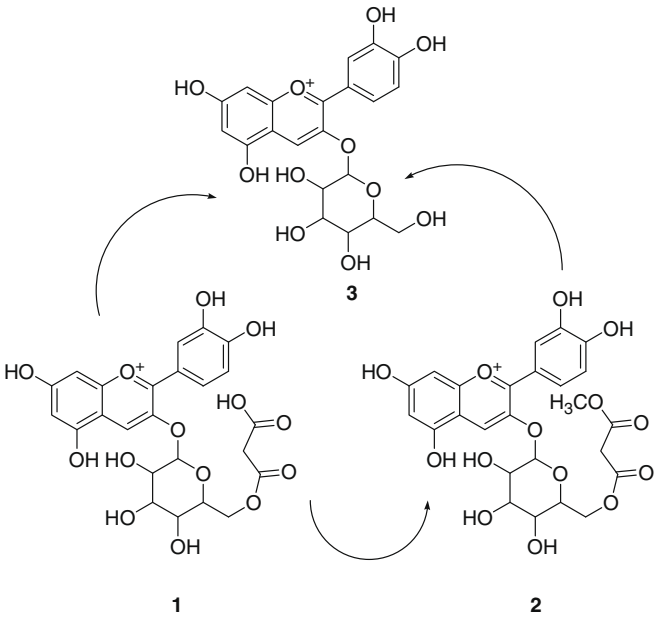

Fig. 4. Structure of anthocyanin **1** (cyanidin-3-*O*-(6″-malonyl-β-D-glucopyranoside), **2** (cyanidin-3-*O*-(6″-malonyl-β-D-glucopyranoside methyl ester) and **3** (cyanidin-3-*O*-β-D-glucopyranoside).

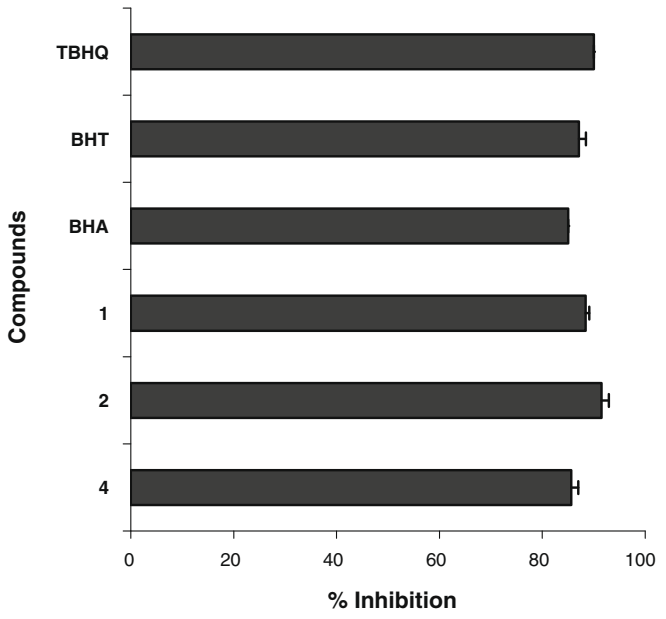

Fig. 5. Lipid peroxidation inhibitory activity of compounds **1** (cyanidin-3-*O*-(6″-malonyl-β-D-glucopyranoside), **2** (cyanidin-3-*O*-(6″-malonyl-β-D-glucopyranoside methyl ester) and **4** (chicoric acid). Compounds **1** and **2** were tested at 0.25 μg/ml. Compound **4** was tested at 50 μM. Positive controls BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene) and TBHQ (t-butyl hydroquinone) were tested at 10 μM concentration. The percent inhibition was calculated with respect to solvent control (DMSO) and values represent means ± SD ($n = 2$). Activity of anthocyanin **3** (cyanidin-3-*O*-β-D-glucopyranoside) was reported earlier from our laboratory (Mulabagal et al., 2007).

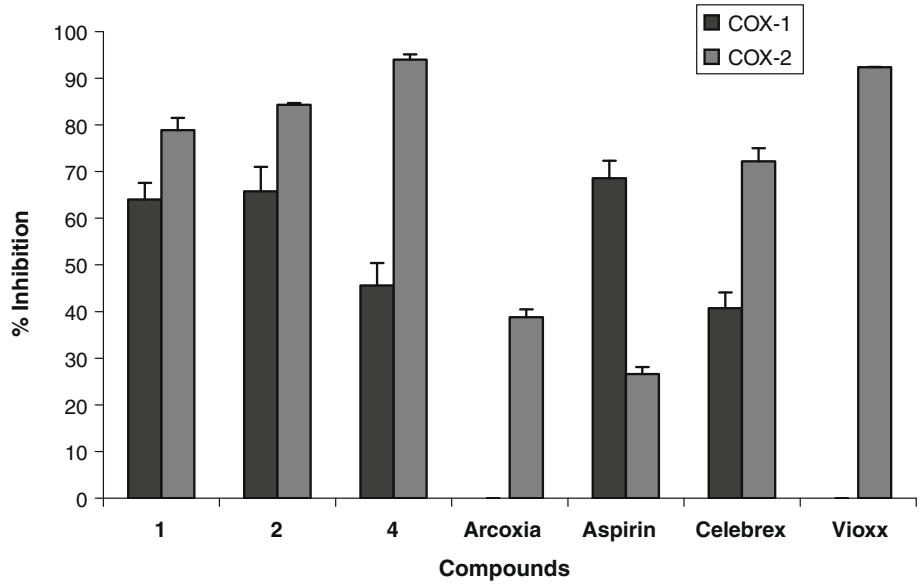

Fig. 6. *In vitro* COX-1 and COX-2 enzyme inhibitory activities of compounds **1** (cyanidin-3-*O*-(6″-malonyl-β-D-glucopyranoside at 0.25 μM), **2** (cyanidin-3-*O*-(6″-malonyl-β-D-glucopyranoside methyl ester at 0.25 μM) and **4** (chicoric acid at 50 μM) and positive controls, Arcoxia (128 nM), Aspirin (60 μM), Celebrex (26 nM) and Vioxx (32 nM). Vertical bars represent the averages of two experiments ± SD. Activity of anthocyanin **3** (cyanidin-3-*O*-β-D-glucopyranoside) was reported earlier from our laboratory (Mulabagal et al., 2007).

and anti-inflammatory properties (Henry, Momin, Nair, & Dewitt, 2002). Isolation of quercetin, caffeic acid, chicoric acid and anthocyanins was reported from other varieties of lettuce (Nicolle & Cardinault et al., 2004; Nicolle & Carnat et al., 2004; Romani et al., 2002). The chicoric acid (**4**), isolated from both green and red lettuce, showed 85.6%, 45.6% and 94% of LPO, COX-1 and -2 enzyme inhibitions at 50 μM, respectively. Other phenolic compounds, monocaffeoyltartaric acid, dicaffeoyltartaric acid, 5-caffeoylquinic acid, caffeoylmalic acid and 3,5-di-*O*-caffeoylquinic acid, have also been reported from lettuce and their contents may vary according to harvesting period (Sobolev, Brosio, Gianferri, & Segre, 2005). This is the first report of the LPO, COX-1 and -2 enzyme inhibitory activities of chicoric acid (**4**) (Figs. 5 and 6).

Most green vegetables contain large quantities of the green pigment chlorophyll. Previous studies from our laboratory have demonstrated that chlorophyll, quercetin and quercetin glucoside inhibited COX-1 and COX-2 enzymes (Reddy, Alexander-Lindo, & Nair, 2005; Vanisree, Alexander-Lindo, DeWitt, & Nair, 2008) *in vitro*. Studies have shown that chicoric acid had antioxidant activity (Thygesen, Thulin, Mortensen, Skibsted, & Molgaard, 2007) and inhibited the replication of HIV virus in tissue culture (Lee, Shin, Lee, & Lee, 2007). From the present study, it is clear that the biological activities observed for the methanolic extracts of red and green lettuce are due to the presence of phenolic compounds.

4. Conclusions

Our results have shown that both red and green lettuce had strong antioxidant and anti-inflammatory activities. However, the anthocyanin in red lettuce may be considered as an additional source of biological activity, based on a number of *in vitro* and *in vivo* experiments suggesting its health-beneficial activities. Our results also demonstrated the difference in biological activities between green and red lettuce due to the variation in phenolic composition. Higher amounts of phenolics, including the anthocyanin, present in red lettuce, may indicate that consumption of red lettuce may provide better health-benefits than green lettuce.

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