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Antioxidant and anti-inflammatory activity of aqueous fraction from *Albizia lebbeck* leaves

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Abstract

The present study used in vitro digestion coupled with Caco-2 cells to assess antioxidant and anti-inflammatory activities of an aqueous fraction from *Albizia lebbeck (A. lebbeck)* young leaves collected from 3 provinces of Thailand i.e.; Suphanburi (SP), Nakhonratchasima (NA) and Kanchanaburi (KA). *A. lebbeck* young leaves from SP had the highest content of flavonoids while those from KA possess the highest of carotenoids. The aqueous fraction of *A. lebbeck* young leaves from SP had the highest antioxidant capacity. Pre-treatment of Caco-2 cells monolayer with the aqueous fraction of *A. lebbeck* young leaves from SP significantly restored intracellular glutathione content and glutathione peroxidase activity induced by H_2O_2+IL-1β. Pre-incubation of Caco-2 cells monolayer with the aqueous fraction of *A. lebbeck* young leaves from 3 provinces significantly decreased secretion of IL-8 and MCP-1. Similar to the antioxidant capacity, the aqueous fraction of *A. lebbeck* young leaves from SP had the highest anti-inflammatory capacity. Pre-treatment of Caco-2 cells monolayer with the aqueous fraction of *A. lebbeck* young leaves from 3 provinces significantly decreased TNF-α and IL-6 production and COX-2 protein expression but there was no statistically difference noted in suppressive effect among samples from 3 provinces. The present data indicated that digested active compounds in *A. lebbeck* young leaves retained their antioxidant and anti-inflammatory capacity and enhanced cytoprotective effects by reducing potential damage caused by oxidative stress and inflammatory insult.

Introduction

Intestinal epithelial cells perform various physiological functions including nutrient absorption and acting as natural barriers against gut microbiota, antigens and xenobiotics. In case of exposure to some adverse condition including tissue damage or infection triggers intestinal cells to secrete inflammatory cyto/chemokines in response against such stimuli (Kagnoff, 2014). During intestinal inflammation, gut homeostasis is disturbed by excessive production of inflammatory mediators which induce the onset of intestinal disorders such as inflammatory bowel diseases (IBDs) (Sonier *et al*., 2009; Wang *et al*., 2009). Inflammatory bowel disease (IBD) including Ulcerative colitis (UC) and Crohn's diseases (CD) are characterized by chronic inflammation of the gastrointestinal tract and thus causes damage to the structure and function of intestinal barrier (Laukoetter *et al*., 2008). The etiology of IBD is not well defined; however, oxidative stress is a potential triggering factor (Gill *et al*., 2010). The intestinal mucosa of IBD patients had greater amounts of reactive oxygen species (ROS), reactive nitrogen species (RNS) and other oxidative stress markers but less quantity of antioxidants than those of control subjects. Alteration of oxidative stress markers was associated with the severity of intestinal inflammation in IBD patients (Zhu and Li, 2012). Overproduction of several proinflammatory mediators during inflammatory response may generate more oxidative products, leading to auto-amplification of vicious cycle to impair the gut barrier thus aggravate inflammatory damage (Biasi *et al*., 2013). Pharmacological treatment of IBD includes anti-inflammatory drugs, such as 5-aminosalicylic acid derivatives, glucocorticoids and biological therapies such as monoclonal antibody of TNF-α (Di Stasi *et al*., 2015). However, long-term usage of pharmacological management of IBD is associated with serious side effects which reduce patient compliance and worsen the disease condition (Fakhoury *et al*., 2014). Biological therapies have also been associated with several adverse effects (Arora and Shen, 2015) and the cost of these agents remains high and ineffective in some patients (D'Haens, 2007) resulting in limiting use of this therapeutic approach. Dietary phytochemicals are of increasing interest as an alternative approach to

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alleviate inflammatory associated symptoms (Boeing *et al*., 2012) due to their relative safety cost-effective.

Albizia lebbeck is a fast growing and medium size deciduous tree in Asia. Its leaves, barks, seeds and roots are used as folk Indian medicine (Faisal *et al.*, 2012). A previous report compiled traditional uses of medicinal plants among the rural communities in India which informed medicinal properties of *A. lebbeck* such as the leaf juice as eye drop for remedy night blindness, decoction of the bark is used as mouth wash to heal gum and the powder root bark is applied externally as plaster to cure leprous ulcers (Parveen *et al*., 2007). Various organic solvent extracts of stem bark of *A. lebbeck* or known as Shirisha in Ayruvedic medicines have been demonstrated to inhibit paw edema inflammation induced by carrageenan, dextran, cotton pellet and Freund's adjuvant (Babu *et al*., 2009). Its leaves have several therapeutic active compounds including alkaloids, flavonoids, tannin and saponins (Chulet *et al.*, 2010). Anti-inflammatory and antioxidant activities of dietary flavonoids have been well documented in several studies (Pan *et al*., 2010; Kumar and Pandey, 2013; Ribeiro *et al*., 2015) and anti-inflammation of saponin was also recently reported (Sun *et al.*, 2015). Due to high content of such bioactive compounds, adequate intake of *A. lebbeck* leaves has potential to provide health benefit. The present study aims to assess anti-inflammatory and antioxidant effect of digestive fraction of *A. lebbeck* leaves grown in three areas of Thailand by using oxidative/cytokine induced human intestinal like Caco-2 cells model. This cell model is a well-established model for evaluation of anti-inflammatory and antioxidant activities of active compounds in foods. Cultures are pre-treated with compounds of interest before insulting the cells with oxidants and/or pro-inflammatory cytokines to assess the possible suppression or activation of the intestinal cellular response against such insults (Taverniti *et al*., 2014; Sangiovanni *et al.*, 2015; Tuntipopipat *et al*., 2015).

Materials and Methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), α-amylase, pepsin, porcine bile extract, porcine pancreatin, porcine lipase and protease from bovine pancreas were purchased from Sigma Chemical Co. (St. Louis, MO, USA). L-glutamine, nonessential amino acids penicillin-streptomycin and fungizone were obtained from Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from PAA Laboratories (GmbH, Pasching, Austria). Human TNF-α, IL-6, IL-8 and MCP-1 capture and biotin-labeled detection antibodies, and human IL-1β was purchased from Peprotech Inc. (Rocky Hill, NJ, USA). Primary antibodies against COX-2 were purchased from Cell Signaling Technology (Danvers, MA, USA). Glutathione assay and glutathione peroxidase activity assay kits were purchased from Cayman (Ann Arbor, Michigan, USA). All chemicals were either analytical grade or HPLC grade.

Sample preparation

Albizia lebbeck (A. lebbeck) young leaves, commonly consumed by Thai population, were collected from 3 provinces: Suphanburi (SP), Nakhonratchasima (NA) and Kanchanaburi (KA) which represent the central, northeast and western areas of Thailand. All the samples collected from each area were extensively washed with deionized water and air dried. Edible portion of young leaves was submerged in boiled water at ratio 1:2 (w/v) for 2 min and air dried prior to homogenization by electric blender and then lyophilized until dryness. Dried samples were stored in aluminum foil in vacuo at -20 ºC.

In vitro *digestion and bioaccessibility*

Simulated digestion was conducted according to Chitchumroonchokchai *et al.* (2004). Digestion reactions involved 0.3 g freeze dried sample with 3% (v/w) soybean oil. After completion of the simulated digestion, chyme was centrifuged (Becton Dickinson Dynac Centrifuge, Sparks, MD, USA) at 10,000 g for 1 h at room temperature to isolate the aqueous fraction. The supernatant after centrifugation was filtered (0.22 μm pores; polytetrafluoroethylene (PTFE) membrane; Millipore Corp., Cork, Ireland) to obtain the fraction with mixed micelles. The filtered aqueous fractions obtained from digested leaves were used to assess antioxidant and anti-inflammatory activity in differentiated human intestinal like Caco-2 cells. Control digestion without induction was also conducted to assess the possible cytotoxicity against Caco-2 cells of active compounds in the aqueous fraction. Bioaccessibility (%) is the amount of flavonoids and carotenoids in the *A. lebbeck* young leaves that were partitioned in the filtered aqueous fraction during simulated digestion to become available for uptake and possible transport across small intestinal absorptive epithelial cells.

Extraction and analysis of carotenoids

Carotenoids in freeze dried sample and the aqueous fraction were extracted and analyzed according to previous protocol (Dawilai *et al.*, 2013). Briefly, freeze dried sample and the aqueous fraction

was dissolved in hexane:acetone:ethanol (50/25/25, v/v/v), sonicated in an ultrasonic bath (Mettler Electronics® Corp., Anaheim, California, USA) for 10 min, and centrifuged at 5,000 g for 10 min. Supernatant was then evaporated with rotary evaporator (Buchi Rotavapor-Re-124, Flawil, Switzerland) at 38-40 °C. The dried extract was resolubilized in 3 mL of mobile phase solution and filtered (through 0.22 µm PTFE membrane). The extract was further diluted to an appropriate concentration with mobile phase prior to analysis by HPLC (Agilent 1100 series, Santa Clara, CA, USA). The concentration of each carotenoid was quantified comparing with a standard containing lutein, zeaxanthin, lycopene, α-carotene and β-carotene.

Extraction and analysis of flavonoids

Flavonoids were extracted and analyzed according to method described by Dawilia *et al.* (2013). Briefly, freeze dried was dissolved in acid methanol containing 0.5 g/L tert-butylhydroquinone with continuous stirring on shaking water bath at 70 °C for 2 h The resulting mixture was placed in cold water bath for 5 min and then added with 100 µl of 1% ascorbic acid solution, followed by volume make up with methanol in 50 mL volumetric flask. The extract was sonicated in ultrasonic bath for 5 min and filtered through 0.2 µm PTFE membrane (Millipore Corp., Cork, Ireland) prior to analysis by HPLC. The aqueous fraction were extracted twice with equal volume of absolute ethanol, sonicated for 10 min, and centrifuged at 5,000 g for 10 min. The combined supernatants were evaporated with rotary evaporator at 38–40°C until dryness. Dried extract was hydrolysed with acid methanol (6 mL deionized water: 4 mL 6 M HCl) and 10 mL methanol containing 0.5 g/L tert-butylhydroquinone (TBHQ) and incubated in a shaking water bath at 70°C for 2 h. Samples were placed on ice for 5 min, added with 1% (w/v) ascorbic acid solution, and brought up to 20 mL with absolute methanol. The extract was sonicated for 5 min and filtered through 0.2 µm PTFE membrane into an amber vial prior to analysis by HPLC (Agilent Technologies 1100 series coupled with a photodiode array detector) which was equipped with a Zorbax EclipaseXDB-C18 column (4.6 x150 mm, inner diameter 5 mm; Agilent Technologies). The mobile phase consisted of 100% water containing 0.5% (w/w) trifluoroacetic acid (solvent A), 100% methanol containing 0.5% (w/w) trifluoroacetic acid (solvent B), and 100% acetonitrile containing 0.5% (w/w) trifluoroacetic acid (solvent C). The flavonoid content was separated by gradient elution programs as previously described by Merken and Beecher (2000) and identified by comparison of retention time and spectra with pure standards (chlorogenic acid, caffeic acid, ferulic acid, taxifolin, vitexin, naringin, naringenin, myricetin, morin hydrate, quercetin, luteolin, hesperitin, kaempferol, apigenin and isorhamnetin). Content was quantified at 338 nm by comparing peak areas with calibration curves.

Oxygen radical absorbance capacity assay (ORAC)

Free radical scavenging activity of aqueous fraction produced from digestion of *A. lebbeck* young leaves was measured by ORAC assay (Ou *et al*., 2001). The ORAC assay is a kinetic assay that measures the loss of fluorescein fluorescence and antioxidant protection over time due to peroxyl-radical formation by the breakdown of 2, 2′-azobis-(2-amidinopropane) dihydrochloride (AAPH). The Trolox, a water soluble vitamin E derivative, serves as a standard and positive control inhibiting fluorescein decay in a dose dependent manner. The fluorescence change of fluorescein can be measured in a time dependent manner. In this assay, a volume of 150 µL of fluorescein (0.0816 µM) was mixed 25 µL of AAPH (153 mM), followed by digested sample (25 µL). For the blank, a volume of 25 µL of phosphate buffer (75 mM) was used instead of digested sample. The loss of fluorescence were measured (excitation, 485 nm; emission, 520 nm) every 5 min over the time period of 90 min using microplate reader (BioTek® Instruments, Vermont, USA). The net area under the curve (AUC) of the blank, standards and samples were estimated. The standard curve was obtained by plotting Trolox concentrations against the average net AUC of the duplicate measurements for each concentration. The final results are expressed as micromole of Trolox equivalent per milligram of sample (µmol TE/mg sample).

Cytotoxicity test

Cells were seeded and maintained in complete medium as previously described by Garrett *et al.* (1999). The cytotoxicity of filtered aqueous fractions from *A. lebbeck* young leaves produced during digestions with H_2O_2+IL-1β activated Caco-2 cells was pre-determined to select the non-toxic concentration for assessment of functional properties. Fully differentiated Caco-2 cells monolayer was incubated with various dilutions of filtered aqueous fractions produced during digestion of leaves for 4 h prior to incubation with 1mM H_2O_2 for 30 min. After washing the monolayer with sterile phosphate buffered saline (PBS), monolayer was stimulated with 10 ng/mL of recombinant human IL-1β for an additional 20 h. Viability of treated cells was assessed

by sulforhodamine B (SRB) assay (Vichai and Kirtikara, 2006). Treated Caco-2 cells monolayer was washed with PBS before initiating the SRB assay. The absorbance wavelength at 500 nm was proportional to cell number. Similarly, activated (H_2O_2 + IL-1β) Caco-2 cells incubated with the diluted aqueous fraction obtained from control digestion (without vegetable) were arbitrarily assigned the value of 100%. Acceptable viability of treated cells was set at >90%.

Anti-inflammatory activity of diluted aqueous fraction on inflamed Caco-2 cells

Experiment was performed on 11^{th} –14^{th} days after Caco-2 cell monolayers become confluent. The monolayer was washed with basal DMEM medium prior to addition of 2 mL of non-toxic diluted fraction or 200 μM quercetin (a well-known antioxidant and anti-inflammation agent). The resulting reaction mixture was incubated for 4 h prior to stimulation with H_2O_2+IL-1b as described in the cytotoxic section. Culture media were collected to measure TNF-α, IL-6, IL-8 and MCP-1 by ELISA (Tuntipopipat et al., 2011; Dawilai *et al.*, 2013). Concentrations of TNF-α, IL-6, IL-8 and MCP-1 were calculated by comparing absorbance with the standard curve of each sample. The treated cells were harvested and cell lysate was prepared according to previous protocol (Tuntipopipat *et al*., 2011). Protein content was determined by a bicinic acid method using bovine serum albumin as a standard. Forty microgram per well of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with COX-2 antibody in Tris-buffered saline containing 0.1% Tween 20 and 5% bovine serum albumin overnight at 4 °C. After washing, membranes were reacted with HRP-conjugated secondary antibody and subsequently incubated with Super Signal solution (Endogen) and exposed to X-ray film. The membranes were reprobed with anti–β-actin to assess the quantity of protein loaded. The density of target bands was quantified by Image J program (Abramoff *et al*., 2004). Results are expressed as relative ratios of band density between the COX-2 and β-actin.

Intracellular reactive oxygen species (ROS) measurement

Caco-2 cells monolayers were treated as described in cytotoxic section. After culture media were collected for the measurement of cytokines, monolayers were washed with warm PBS prior to incubation with 5 μM dichlorofluorescine diacetate (DCF-DA) at 37°C for 30 min. Thereafter, monolayers were subsequently washed with PBS and lysed with 0.5% Triton X-100 in cold PBS before centrifugation at 14,000 g for 5 min at 4°C. The fluorescence intensity of the supernatant was determined using an excitation wavelength of 485 nm and an emission wavelength of 530 nm with a microplate reader.

Measurement of intracellular glutathione (GSH)

Glutathione is a tripeptide of γ-glutamylcysteinylglycine (Foyer *et al*., 1994). It serves as nucleophilic co-substrate to glutathione transferases in xenobiotic detoxification and is an essential electron donor to glutathione peroxidases in the reduction of hydroperoxides (Baillie and Slatter, 1991). Intracellular glutathione content in Caco-2 cells was measured by glutathione assay kit (Cayman Chemical Co., Ann Arbor, MI). The concentration of total glutathione was measured by the enzymatic recycling method according to the manufacturer's protocol. Briefly, cells were washed with cold PBS and harvested by scraping into cold PBS and sonicated on ice. The cell homogenate was centrifuged at 13,500 g for 10 min at 4°C. The supernatant incubated at room temperature for 5 min with 10% metaphosphoric acid (v/v). The mixture was centrifuged (15,000 g, 5 min) and the supernatant analyzed for total glutathione by the GSH disulphide reductase 5, 5′-dithiobis (2-nitrobenzoic acid) recycling method using the procedure described in the glutathione assay kit.

Measurement of intracellular glutathione peroxidase activity

Glutathione peroxidase catalyzes the reduction of hydroperoxides by reduced glutathione and functions to protect the cell from oxidative damage. The enzyme utilizes glutathione as the ultimate electron donor to regenerate the reduced form of the selenocysteine in subunit of glutathione peroxidase (Ursini *et al.*, 1985). Intracellular glutathione peroxidase activity was measured by glutathione peroxidase assay kit (Cayman Chemical). Briefly, cells were washed with cold PBS and harvested by scraping into cold PBS and then sonicated at 0°C. The cell homogenate was centrifuged at 13,500 g for 10 min at 4°C. The supernatant was analyzed for glutathione peroxidase activity using the procedure described in the test kit.

Data analyses

SPSS version 14 was used for statistical analyses. All parameters were conducted in triplicate and each

experiment was performed at least 2 times. Data are presented as means ± standard deviation (SD). Differences between group means were determined using One-way ANOVAs, followed by Tukey posthoc analysis. Data are considered significant at $p < 0.05$.

Results

Quantity of carotenoids and flavonoids

The flavonoids and carotenoid content of *A. lebbeck* young leaves from SP, NA and KA were presented in Table 1. Quercetin was the predominant flavonoid followed by luteolin, kaempferol and apigenin. *A. lebbeck* young leaves from SP had the highest content of total flavonoids (quercetin, kaempferol, luteolin and apigenin) followed by those from KA and NA, respectively. Lutein was a predominant carotenoid followed by β-carotene and α-carotene in *A. lebbeck* young leaves. *A. lebbeck* young leaves from KA contained total carotenoid content 2 times greater than those from SP and NA (Table1).

Bioaccessibility of flavonoids and carotenoids

The relative efficiency with which quercetin (approx. 66%-68%), kaempferol (approx. 60%-62%), luteolin (approx. 51%-52%) and apigenin (approx. 28%-30%) of *A. lebbeck* young leaves from SP, NA and KA were transferred to the aqueous fraction of chyme was not affected by differences among the three sources of *A. lebbeck* young leaves (Table 2). The actual amount of quercetin in the aqueous fraction (bioaccessible fraction) of digested *A. lebbeck* young leaves from SP was significantly greater than those from NA and KA while the aqueous fraction of digested *A. lebbeck* young leaves from NA contained a greater actual content of luteolin than ($p<0.05$) those from SP and KA (Table 2). The aqueous fraction of digested *A. lebbeck* young leaves from KA contained the highest content of kaempferol and apigenin followed by those from SP and NA, respectively (Table 2). Percent bioaccessibility of lutein (approx. 35%-39%) and β-carotene (approx. 24%-27%) of digested *A. lebbeck* young leaves from SP and NA was significantly greater than those from KA, while percent bioaccessibility of α-carotene (approx. 25%-27%) of digested *A. lebbeck* young leaves among 3 provinces did not differ significantly (Table 2).The amount of total carotenoids in aqueous fraction of digested *A. lebbeck* young leaves in KA was 2 times greater than that of SP and NA.

Table 1. Flavonoid and carotenoid contents of aqueous fraction from *A. lebbeck* leaves collected from SP, NA and KA (mean ± SD; n=6) (mg %dry weight

Flavonoids	SP	NA	KA
Quercetin	673 ± 22^a	498 ± 14^c	565 ± 25^b
Kaempferol	32.2 ± 2.1^b	10.6 ± 0.2^c	48.8 ± 2.4^a
Luteolin	148 ± 3^a	241 ± 8^b	162 ± 5^a
Apigenin	12.3 ± 0.9^b	8.2 ± 0.3^c	18.3 ± 1.3^a
Total	**865.5 ± 28.5**	**757.8 ± 22.5**	**794.1 ± 33.7**
Carotenoids	SP	NA	KA
Lutein	25.0 ± 0.7^b	23.1 ± 1.1^b	53.4 ± 0.7^a
α-carotene	1.9 ± 0.1^b	1.3 ± 0.1^c	2.7 ± 0.1^a
β-carotene	11.5 ± 0.2^b	9.9 ± 0.4^c	20.3 ± 0.8^a
Total	**38.4 ± 1.0**	**34.3 ± 1.6**	**76.4 ± 1.6**

Different letters next to each mean ± SD denote a significant difference ($p<0.05$)

Aqueous fraction of A. lebbeck young leaves suppressed chemo/cyto-kines secretion and COX-2 expression by activated Caco-2 cells

The diluted filtered aqueous fraction (1:3) of *A. lebbeck* young leaves did not show cytotoxic effect as assessed by SRB assay. Caco-2 cell monolayer was incubated with the diluted filtered aqueous fraction of *A. lebbeck* young leaves for 4 h prior to addition of H_2O_2 +IL-1β to cultures for an additional 20 h (data not shown). Incubation of Caco-2 cells with H_2O_2+IL-1β secreted 500-fold and 15-fold more IL-8 and MCP-1 into medium than control cultures ($p<0.05$) (Figure 1A and 1B). Pre-incubation of Caco-2 cells monolayer with 200 µM quercetin (a positive control) significantly suppressed IL-8 and MCP-1 secretion by 46% and 47%, respectively (Figure 1A and 1B). Pre-incubation of Caco-2 cells monolayer with the diluted filtered aqueous fraction of *A. lebbeck* young leaves decreased secretion of IL-8 and MCP-1 by 32%-37% and 33%-37% ($p<0.05$), respectively (Figure 1A and 1B). Sample from SP exhibited a significantly greater suppressive effect on IL-8 and MCP-1 secretion than sample from the other two provinces ($p<0.05$). Similarly, activation of Caco-2 cells with H_2O_2 +IL-1β following pre-treatment with diluted filtered aqueous fraction lacking *A. lebbeck* young leaves significantly secreted 4.5-fold and 4.4-fold more TNF-α and IL-6 into medium than control cultures (Figure 1). Incubation of Caco-2 cells monolayer with 200 µM quercetin (food derived well-known antioxidant) prior to activation with H_2O_2 +IL-1β significantly decreased the secretion

Table 2. Percent bioaccessibility and content of flavonoids and carotenoids (µg/g dry weight) in bioaccessible fraction of *A. lebbeck* leaves from SP, NA and KA

Compounds	% Bioaccessibility			Amount in bioaccessible fraction		
	SP	NA	KA	SP	NA	KA
Quercetin	66.7 ± 4.0^{a}	68.7 ± 3.6^{a}	66.0 ± 2.4^{a}	446.2 ± 26.7^{a}	339.1 ± 17.7^{b}	369.7 ± 13.4^{b}
Kaempferol	61.5 ± 3.0^{a}	60.4 ± 6.6^{a}	62.6 ± 3.6^{a}	19.5 ± 0.9^{b}	6.2 ± 0.7^{c}	29.7 ± 1.7^{a}
Luteolin	52.0 ± 4.1^{a}	51.1 ± 1.5^{a}	51.3 ± 3.2^{a}	76.1 ± 6.0^{b}	118.6 ± 3.4^{a}	78.7 ± 4.9^{b}
Apigenin	29.3 ± 1.4^{a}	29.9 ± 2.6^{a}	28.6 ± 1.2^{a}	3.4 ± 0.2^{b}	2.4 ± 0.2^{c}	4.9 ± 0.2^{a}
Lutein	38.6 ± 2.5^{ab}	39.6 ± 1.4^{a}	35.7 ± 2.3^{b}	10.0 ± 0.6^{b}	9.35 ± 0.3^{b}	18.7 ± 1.2^{a}
α-carotene	26.9 ± 2.3^{a}	25.8 ± 1.0^{a}	25.0 ± 2.4^{a}	0.50 ± 0.04^{b}	0.32 ± 0.01^{c}	0.65 ± 0.06^{a}
β-carotene	25.8 ± 1.3^{ab}	27.1 ± 1.8^{a}	24.5 ± 1.2^{b}	3.02 ± 0.1^{b}	2.56 ± 0.2^{c}	5.0 ± 0.2^{a}

Data are the mean ± SD; n=6. Different letters in the same row next to each mean ± SD of % bioaccessibility and amount in bioaccessible fraction denote a significant difference ($p<0.05$)

of TNF-α and IL-6 by 40% and 55%, respectively (Figure 1A and 1B). Pre-treatment of Caco-2 cells monolayer with the diluted filtered aqueous fraction of *A. lebbeck* young leaves from 3 sources before induction with H_2O_2 +IL-1β significantly decreased the TNF-α and IL-6 production by 32%-37% and 25%-30%, respectively (Figure 1C and 1D). Caco-2 cells monolayer significantly expressed COX-2 protein when cells were stimulated with H_2O_2 +IL-1β (Figure 1E). Prior incubation of Caco-2 cell monolayer with quercetin suppressed activated COX-2 protein by 46% ($p<0.05$) (Figure 1E). Likewise, incubation of Caco-2 cells with the diluted filtered aqueous fraction of *A. lebbeck* young leaves prior to activating the monolayer with H_2O_2+IL-1β attenuated the COX-2 expression by 25%-28% ($p<0.05$) (Figure 1E). However, there was no statistical significant suppression noted in TNF-α, IL-6 production and COX-2 expression among the samples used from three provinces.

Antioxidant capacity of aqueous fraction from A. lebbeck *young leaves*

The present study used two methodologies for analysis of antioxidant activities by determining ROS scavenging potentials through the assessment of the peroxyl radical scavenging activity in the cell-free oxygen radical antioxidant capacity (ORAC) assay and the cell-based antioxidant activity (CAA) assay (Wolfe and Rui, 2007). As shown in Figure 2A, aqueous fraction of *A. lebbeck* young leaves from SP had the highest ORAC value whilst KA and NA had about 10%-20% and 10% lower antioxidant capacity than SP ($p<0.05$). Incubation of Caco-2 cells monolayer with H_2O_2+IL-1β enhanced 4.7-fold more intracellular ROS formation than control cultures (Figure 2B). Pre-incubation of Caco-2 cells monolayer with quercetin significantly suppressed H_2O_2+IL-1β induced ROS formation by 40% (Figure 2B). Pre-treatment of Caco-2 cells monolayer with the diluted filtered aqueous fraction of *A. lebbeck* young leaves significantly attenuated H_2O_2+IL-1β induced ROS levels by 20%-25% (Figure 2B). The *A. lebbeck* young leaves from SP had the highest potency (Figure 2B).There was a high correlation between antioxidant capacity expressed by Trolox equivalent (ORAC) and suppressive capacity of intracellular ROS ($r^2=0.96$, $p<0.01$)

Aqueous fraction of A. lebbeck *young leaves enhanced glutathione content and glutathione peroxidase activity in Caco-2 cells*

Incubation of Caco-2 cells monolayer with H_2O_2 +IL-1β significantly depleted intracellular glutathione by 56% of basal content in control cultures (Figure 3). Prior incubation Caco-2 cells monolayer with quercetin significantly repleted H_2O_2+IL-1β induced intracellular glutathione content (Figure 3). Pre-treatment Caco-2 cells monolayer with the diluted filtered aqueous fraction of *A. lebbeck* young leaves from SP significantly (Figure 3A) restored intracellular glutathione content induced by H_2O_2 +IL-1β, whilst those from NA did not show significant effect (Figure 3A). Similarly, incubation of Caco-2 cells monolayer with H_2O_2 +IL-1β significantly decreased glutathione peroxidase activity by 46% of basal content in control cultures (Figure 3B). Pretreatment Caco-2 cells with quercetin significantly enhanced the depleted glutathione peroxidase activity induced by H_2O_2+IL-1β (Figure 3B). However, the diluted filtered aqueous fraction of *A. lebbeck* young leaves from SP and NA significantly (Figure 3B) repleted intracellular glutathione peroxidase activity (Figure 3B) whilst that of KA did not produce any significant impact on

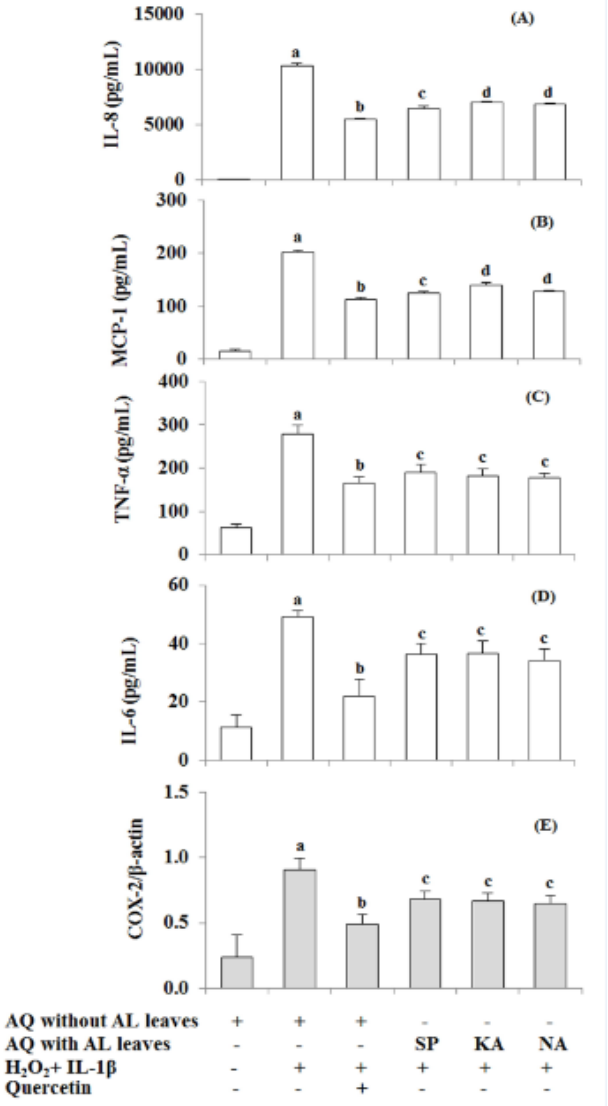

Figure 1. Pre-treatment of Caco-2 cells with the aqueous fraction (AQ) fraction of digested *A. lebbeck* (AL) leaves suppresses the secretion of cyto/chemo-kines and COX-2 expression in response against H_2O_2 + IL-1β. Differentiated Caco-2 cells monolayer were incubated for 4 h either with control medium (bars 1 and 2) or with the aqueous fraction of digested AL from Suphanburi (SP; bar 4) or Kanchanaburi (KA; bar 5) or Nakhonratchasima (NA; bar 6) or Quercetin (bar 3). Medium was removed after 4 h before the addition of a fresh medium without (control; bar 1) or with H_2O_2 + IL-1β (bar 2-6) Medium and cell lysate were collected after 20 h to measure IL-8 (A), MCP-1 (B), TNF-α (C), IL-6 (D) and COX-2 (E). Data represent mean ± SD for at least 6 replicate cultures. Different letters above the error bars indicate that the mean quantities for the indicated treatments differ significantly ($p < 0.05$).

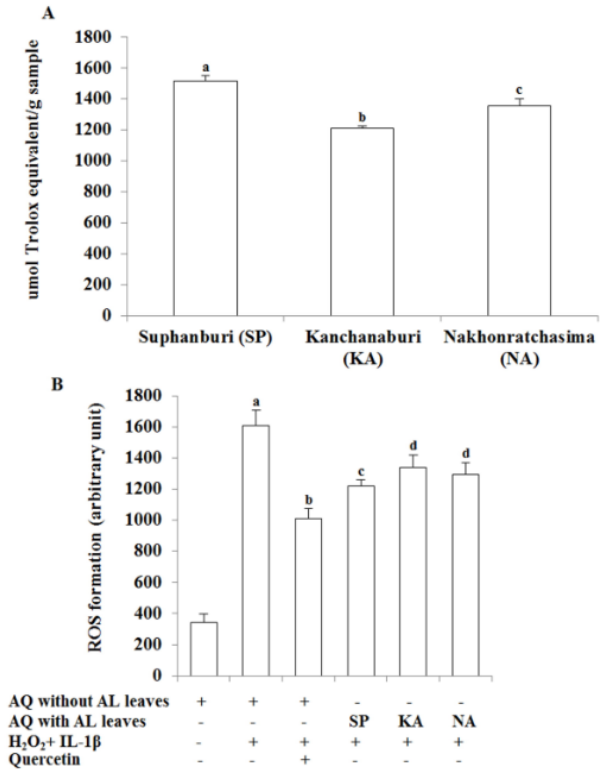

Figure 2. Antioxidant activity of aqueous fraction from *A. lebbeck* (AL) leaves. (A) Free radical scavenging activity of aqueous fraction of AL leaves from Suphanburi (SP), Kanchanaburi (KA), Nakhonratchasima (NA) was measured by ORAC. (B) Intracellular ROS produced by Caco-2 cells exposed to H_2O_2 + IL-1β is decreased by pre-treatment with the aqueous fraction of *A. lebbeck* (AL) leaves. Caco-2 cells were treated as described in the legend of Fig. 1. Medium with DCF-DA was added to the washed monolayers to measure the intracellular ROS. Data represent mean ± SD for at least 6 replicates. Different letters above the error bars indicate that the mean differ significantly ($p < 0.05$).

glutathione peroxidase activity (Figure 3B).

Discussion

Phytochemicals content are affected not only by climatic conditions but also geographical condition. The present study analyzed flavonoids and carotenoids content of *A. lebbeck* young leaves collected from 3 different areas of Thailand. *A. lebbeck* young leaves grown in SP province possessed greater content of total flavonoids while KA had greater carotenoids content than the samples from the other two provinces (Table 1). This finding was coincidental with the bilberries which were grown in high photosynthetic active radiation contained higher levels of anthocyanins, flavonols and hydroxycinnamic acids and lower levels of organic acids compared with bilberry fruit from low-light locations (Mikulic-Petkovsek *et al*., 2015). Glycoside of kaempferol and quercetin were previously identified in *A. lebbec*k young leaves (El-Mousallamy, 1998). Besides flavonoids, tannins and saponins were also found in the solvent extract from *A. lebbeck* young leaves (Chulet *et al*., 2010). However, carotenoids content from *A. lebbeck* young leaves had never been reported in any previous studies.

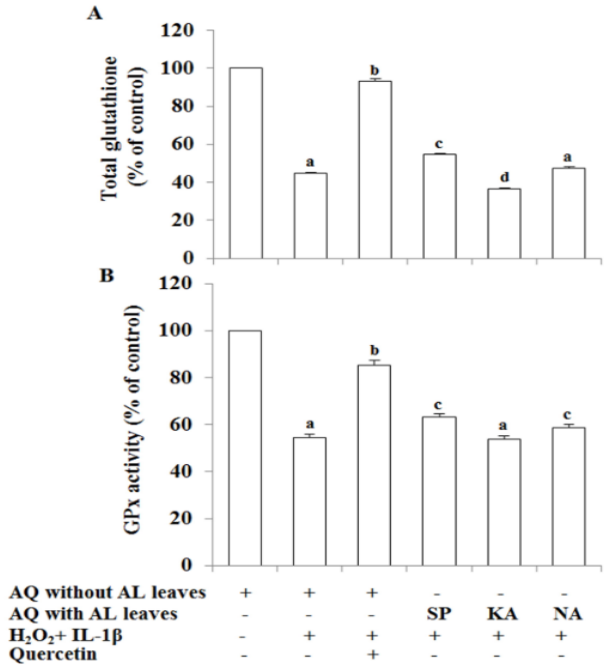

Figure 3. The aqueous fraction (AQ) fraction of digested *A. lebbeck* (AL) leaves repletes intracellular glutathione content and glutathione peroxidase activity (GPx) in H_2O_2 + IL-1β induced Caco-2 cells. Differentiated Caco-2 cells monolayer was treated as described in legend of Figure 1. After culture media were removed, lysates from treated cells were quantified total glutathione content (A) and GPx activity (B). Data are means ± SD of at least 6 replicate cultures. Different letters above the error bars indicate that the mean quantities for the indicated treatments differ significantly ($p < 0.05$).

The transfer of flavonoids from *A. lebbeck* young leaves to the aqueous fraction during digestion was relatively efficient (~30%-69%). These results agreed with a previous report showing 48% of kaempferol in the digested *Eryngium foetidum* leaves were present in the filtered aqueous fraction (Dawilai *et a*l., 2013). Carotenoids 24%-40% in *A. lebbeck* young leaves were transferred to the micelle fraction which is in agreement with a previous report that found 24% β-carotene and 35% lutein in the digested *E. foetidum* leaves were present in the micelle fraction (Dawilai *et al*., 2013).

Antioxidant and anti-inflammatory capacities of flavonoids (Ribeiro *et al*., 2015) and carotenoids (Kaulmann and Bohn 2014) were well-documented. The aqueous fraction of *A. lebbeck* young leaves from SP province had a greater actual content of flavonoids than samples from the other two provinces (Table 2). In agreement with the actual content of flavonoids in the aqueous fraction, the free radical scavenging activity and suppressive ROS formation by aqueous fraction of *A. lebbeck* young leaves from SP was significantly higher than that of NA and KA (Figure 2A-2B). In addition, the aqueous fraction of digested A. lebbeck young leaves from SP exhibited a greater capacity to replete the intracellular glutathione content and glutathione peroxidase activity (GPx) in H_2O_2 + IL-1β induced Caco-2 cells than that from KA (Figure 3). Similarly, the aqueous fraction of digested *A. lebbeck* young leaves from SP showed more suppressive effect on IL-8 and MCP-1 than samples from the other two provinces (Figure 1). Although the aqueous fraction of digested *A. lebbeck* young leaves from KA contained greater carotenoids content than samples from other two provinces, its antioxidant and anti-inflammatory capacities did not get along with the carotenoids content. These data indicated that flavonoids in *A. lebbeck* young leaves play major role to suppress oxidative stress and inhibit IL-8 and MCP-1 secretion in the present study, while both flavonoids and carotenoids in the aqueous fraction of digested *A. lebbeck* young leaves suppress TNF-α, IL-6 secretion and COX-2 expression.

Oxidative/cytokine induced human intestinal like Caco-2 cells used in the present study is a widely used *in vitro* model to mimic conditions of an in *vivo* inflammatory conditions (Shin *et al*., 2015). Uncontrolled inflammatory immune responses play a pivotal role in pathogenesis of Ulcerative colitis (UC) and CD (Mitsuyama *et al*., 2005). In general, active inflammation occurs in the intestinal mucosa of UC patients and migration of neutrophils from blood circulation into intestinal mucosa is the key pathology of tissue inflammation in UC. IL-8, a chemokine, plays role in the activation and recruitment of neutrophils to the inflamed tissues (Baggiolini *et al*., 1989). IL-8 concentration of distal colon from UC patients showed a positive correlation with disease activity score observed by endoscopy and histology (Pearl *et al*., 2013). Suppression of mucosal IL-8 expression was shown to significantly improve the histology of the colon from UC patients receiving partition-herb moxibustion (Zhou *et al*., 2009). MCP-1 is another chemokine playing role to recruit and activate monocytes and macrophages to the inflamed sites (Matsushima *et al*., 1989). Several clinical studies and experimental IBD have shown the up regulation of MCP-1 in mucosal tissues (Reinecker *et al*., 1995; Mazzucchelli *et al*., 1996). Critical role of MCP-1 in the pathogenesis of experimental colitis was investigated in hapten-induced experimental colitis in MCP-1 deficient mice (Khan *et al*., 2006). A significant reduction in the colitis severity observed by macroscopically and histologically with a mortality reduction in MCP-1-deficient mice compared with wild-type. Another study found that mice orally received bindarit (an inhibitor of MCP-1 production) at 100 mg/Kg

reduced clinical and histopathological severity of trinitro-benzene sulfonic acid induced colitis. These effects were associated with significant inhibition of MCP-1 and myeloperoxidase in colon extracts. These results provide evidence for a critical role of IL-8 and MCP-1 in the development of colonic inflammation.

Circulating TNF-α level and colonic TNF-α was increased in children with colonic CD (Murch *et al*., 1991; Breese *et al*., 1994) and overexpression of TNF-α was observed in CD and UC biopsies (Reinecker *et al.*, 1993). As a result, developments of anti-TNF antibodies are increasing interest for IBD treatment (Rutgeerts *et al*., 2005). Data from meta-analyses suggested that Infliximab, a chimeric monoclonal antibody against TNF-α, had a significant effect in induction of clinical response of UC while adalimumab, a human monoclonal antibody against TNF-α, had a significant effect in induction of clinical remission and clinical response of UC compared with conventional therapy (Zhou et al., 2015). Besides TNF-α, serum IL-6 was also significantly increased in IBD patients in comparison to healthy controls (Gross *et al.,* 1992) and the expression extent of IL-6 was associated with clinical, endoscopical, and histopathological severity in both CD and UC patients (Hyams *et al*., 1993). Reduction of serum IL-6 levels correlated with the improvement of disease activity after anti-inflammatory therapy (Umehara *et al.*, 2006). These data support the important role of TNF-α and IL-6 on pathogenesis resulting in inflammatory response of IBD patients.

Cyclooxygenase-2 (COX-2), a proinflammatory inducible enzyme, was also up regulated in inflamed tissue in IBD patients (Singer *et al*., 1998). Pan-colitis in chronic IBD significantly increases the risk of colorectal (Breynaert *et al*., 2008). Patients suffering from long standing IBD face an increased lifetime risk of developing cancer (CRC). COX-2 immunostaining biopsies taken during colonoscopy was positive (total score ≥3) in 72.5% of malignancy long-standing ulcerative colitis (Fratila and Ilias, 2013). Regular use of nonselective nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors (COXIBs) over a 10- to 15-year period reduced the relative risk of CRC development by 40–50% in several studies (Flossmann and Rothwell, 2007; Chan *et al*., 2008; Rostom *et al*., 2007). These data describe the role of COX-2 associated with chronic colitis.

The present data clearly showed that the aqueous fractions obtained from digested *A. lebbeck* young leaves from 3 provinces of Thailand significantly suppressed IL-8, MCP-1, TNF-α, IL-6 and COX-2 producing by H_2O_2+IL-1β induced Caco-2 cells. Thus, consumption of *A. lebbeck* young leaves may contribute to suppress adverse effect of inflammatory response in patients suffering from colitis.

Intestinal mucosa of either ulcerative colitis or Crohn's disease patients had a greater ROS/RNS formation and biomarkers of oxidative injury, including lipid peroxidation products and protein modifications than those of normal persons (Cracowski *et al*., 2002; Hatsugai *et al*., 2010). On the other hand, the levels of glutathione, coenzyme Q10, glutathione S-transferase (GST), superoxide dismutase, catalase, paraoxonase-1 and metallothionein of intestinal mucosal from IBD patients were significantly lower than those of control subjects (Kruidenier and Verspaget, 2002; Rezaie *et al*., 2007). Moreover, addition of N-acetylcysteine (a well-known antioxidant compound) to conventional mesalamine therapy increased the remission rate in patients with ulcerative colitis (Guijarro *et al*., 2008). Consumption of active compound from turmeric (curcumin) ameliorated severity of disease in active ulcerative colitis and reduced clinical relapse in quiescent ulcerative colitis (Hanai and Sugimoto, 2009). The aqueous fractions produced from digested *A. lebbeck* young leaves from 3 provinces of Thailand significantly reduced intracellular ROS formation producing by H_2O_2+IL-1β induced Caco-2 cells. A combination of currently used pharmaceutics with consumption of *A. lebbeck* young leaves may be an alternative design of novel anti-Inflammatory Bowel Diseases therapies.

Conclusion

The present data indicated that *A. lebbeck* young leaves retain antioxidant and anti-inflammatory activities after digestion. The aqueous fraction generated during digestion of *A. lebbeck* young leaves collected from different geographical locations contained different actual content of total flavonoids and carotenoids. The potency of antioxidant capacity of *A. lebbeck* young leaves was related to the actual flavonoids content in the aqueous fraction, while both flavonoids and carotenoids in the aqueous fraction of digested *A. lebbeck* young leaves exerted anti-inflammatory capacities. However, other active compounds in *A. lebbeck y*oung leaves may contribute to exert antioxidant and anti-inflammatory activities in the present study. Due to high antioxidant and anti-inflammatory potency of digested *A. lebbeck* young leaves, these functional activities should be confirmed in colitis patients.

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References

Abramoff, M.D., Magalhaes, P.J. and Ram S.J. 2004. Image processing with Image J. Biophotonics International 11(7): 36-42.

Arora, Z. and Shen, B. 2015. Biological therapy for ulcerative colitis. Gastroenterology Report 3(2): 103-109.

Babu, N. P., Pandikumar, P. and Ignacimuthu, S. 2009. Anti-inflammatory activity of Albizia lebbeck Benth., an ethnomedicinal plant, in acute and chronic animal models of inflammation. Journal of Ethnopharmacology 125(2): 356-360.

Baggiolini, M., Walz, A. and Kunkel, S. 1989. Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. Journal of Clinical Investigation 84(4): 1045-1049.

Baillie, T. A. and Slatter, J. G. 1991. Glutathione: a vehicle for the transport of chemically reactive metabolites in vivo. Accounts of Chemical Research 24(9): 264-270.

Biasi, F., Leonarduzzi, G., Oteiza, P. I. and Poli, G. 2013. Inflammatory bowel disease: mechanisms, redox considerations, and therapeutic targets. Antioxidants and Redox Signaling 19(14): 1711-1747.

Boeing, H., Bechthold, A., Bub, A., Ellinger, S., Haller, D., Kroke, A., Leschik-Bonnet, E., Müller, M., Oberritter, H., Schulze, M., Stehle, P. and Watzl B. 2012. Critical review: vegetables and fruit in the prevention of chronic diseases. European Journal of Nutrition 51(6): 637-663.

Breese, E., Michie, C., Nicholls, S., Murch, S., Williams, C., Domizio, P., Walker-Smith, J. and MacDonald, T. 1994. Tumor necrosis factor-alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. Gastroenterology 106(6): 1455-1466.

Breynaert, C., Vermeire, S., Rutgeerts, P. and Van, A. G. 2008. Dysplasia and colorectal cancer in inflammatory bowel disease: a result of inflammation or an intrinsic risk? Acta Gastro-Enterologica Belgica 71(4): 367-372.

Chan, A. T., Giovannucci, E. L., Meyerhardt, J. A., Schernhammer, E. S., Wu, K. and Fuchs, C. S. 2008. Aspirin dose and duration of use and risk of colorectal cancer in men. Gastroenterology 134(1): 21-28.

Chitchumroonchokchai, C., Schwartz, S. J. and Failla, M. L. 2004. Assessment of lutein bioavailability from meals and a supplement using simulated digestion and Caco-2 human intestinal cells. Journal of Nutrition 134(9): 2280-2286.

Chulet, R., Joseph, L., George, M. and Pradhan, P. 2010. Pharmacognostic standardization and phytochemical screening of Albizzia lebbeck. Journal of Chemical and Pharmaceutical Research 2(1): 432-443.

Cracowski, J. L., Bonaz, B., Bessard, G., Bessard, J., Anglade, C. and Fournet, J. 2002. Increased urinary F2-isoprostanes in patients with Crohn's disease. The American Journal of Gastroenterology 97(1): 99-103.

D'Haens, G. 2007. Risks and benefits of biologic therapy for inflammatory bowel diseases. Gut 56(5): 725-732.

Dawilai, S., Muangnoi, C., Praengamthanachoti, P. and Tuntipopipat, S. 2013. Anti-inflammatory activity of bioaccessible fraction from *Eryngium foetidum* Leaves. BioMed Research International 2013:8.

Di Stasi, L. C., Costa, C. A. and Witaicenis, A. 2015. Products for the treatment of inflammatory bowel disease: a patent review (2013 – 2014). Expert Opinion on Therapeutic Patents 25(6): 629-642.

El-Mousallamy, A. M. D. 1998. Leaf flavonoids of *Albizia lebbeck*. Phytochemistry 48(4): 759-761.

Faisal, M., Singh, P. and Irchhaiya, R. 2012. Review of *Albizia lebbeck* a potent herbal drug. International Research Journal of Pharmacy 3: 63-68.

Fakhoury, M., Negrulj, R., Mooranian, A. and Al-Salami, H. 2014. Inflammatory bowel disease: clinical aspects and treatments. Journal of Inflammation Research 7: 113-120.

Flossmann, E. and Rothwell, P. M. 2007. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomized and observational studies. The Lancet 369(9573): 1603-1613.

Foyer, C. H., Lelandais, M. and Kunert, K. J. 1994. Photooxidative stress in plants. Physiologia Plantarum 92(4): 696-717.

Fratila, O. and Ilias, T. 2013. COX-2 and Ki-67 immunohistochemical markers in the assessment of long-standing ulcerative colitis associated dysplasia. Romanian Journal of Morphology and Embryology 54(1): 143-149.

Garrett, D. A., Failla, M. L. and Sarama, R. J. 1999. Development of an *in vitro* digestion method to assess carotenoid bioavailability from meals. Journal of Agricultural and Food Chemistry 47(10): 4301-4309.

Gill, R., Tsung, A. and Billiar, T. 2010. Linking oxidative stress to inflammation: Toll-like receptors. Free Radical Biology and Medicine 48(9): 1121-1132.

Gross, V., Andus, T., Caesar, I., Roth, M. and Schölmerich, J. 1992. Evidence for continuous stimulation of interleukin-6 production in Crohn's disease. Gastroenterology 102(2): 514-519.

Guijarro, L., Mate, J., Gisbert, J., Perez-Calle, J., Marin-Jimenez, I., Arriaza, E., Olleros, T., Delgado, M., Castillejo, M., Prieto-Merino, D., Gonzalez, L. V. and Pena, A. 2008. N-acetyl-L-cysteine combined with mesalamine in the treatment of ulcerative colitis: randomized, placebo-controlled pilot study. World Journal of Gastroenterology 14(18): 2851-2857.

Hanai, H. and Sugimoto, K. 2009. Curcumin has bright prospects for the treatment of inflammatory bowel disease. Current Pharmaceutical Design 15(18): 2087-2094.

Hatsugai, M., Kurokawa, M., Kouro, T., Nagai, K., Arito, M., Masuko, K., Suematsu, N., Okamoto, K., Itoh, F. and Kato, T. 2010. Protein profiles of peripheral blood mononuclear cells are useful for differential diagnosis of ulcerative colitis and Crohn's disease. Journal of Gastroenterology 45(5): 488-500.

Hyams, J. S., Fitzgerald, J. E., Treem, W. R., Wyzga, N. and Kreutzer, D. L. 1993. Relationship of functional and antigenic interleukin 6 to disease activity in inflammatory bowel disease. Gastroenterology 104(5): 1285-1292.

Kagnoff, M. 2014. The intestinal epithelium is an integral component of a communications network. Journal of Clinical Investigation 124(7): 2841-2843.

Kaulmann, A. and Bohn, T. 2014. Carotenoids, inflammation, and oxidative stress—implications of cellular signaling pathways and relation to chronic disease prevention. Nutrition Research 34(11): 907-929.

Khan, W. I., Motomura, Y., Wang, H., El-Sharkawy, R. T., Verdu, E. F., Verma-Gandhu, M., Rollins, B. J. and Collins, S. M. 2006. Critical role of MCP-1 in the pathogenesis of experimental colitis in the context of immune and enterochromaffin cells. American Journal of Physiology - Gastrointestinal and Liver Physiology 291(5): G803-G811.

Kruidenier, L. and Verspaget, H. W. 2002. Oxidative stress as a pathogenic factor in inflammatory bowel disease — radicals or ridiculous? Alimentary Pharmacology and Therapeutics 16(12): 1997-2015.

Kumar, S. and Pandey, A. K. 2013. Chemistry and biological activities of flavonoids: An overview. The Scientific World Journal 2013: 1-16.

Laukoetter, M., Nava, P. and Nusrat, A. 2008. Role of the intestinal barrier in inflammatory bowel disease. World Journal of Gastroenterology 14(3): 401-407.

Matsushima, K., Larsen, C., DuBois, G. and Oppenheim, J. 1989. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. Journal of Experimental Medicine 169(4): 1485-1490.

Mazzucchelli, L., Hauser, C., Zgraggen, K., Wagner, H., Hess, M., Laissue, J. and Mueller, C. 1996. Differential in situ expression of the genes encoding the chemokines MCP-1 and RANTES in human inflammatory bowel disease. The Journal Pathology 178(2): 201-206.

Merken, H. M. and Beecher, G. R. 2000. Liquid chromatographic method for the separation and quantification of prominent flavonoid aglycones. Journal of Chromatography A 897(1-2): 177-184.

Mikulic-Petkovsek, M., Schmitzer, V., Slatnar, A., Stampar, F. and Veberic, R. 2015. A comparison of fruit quality parameters of wild bilberry (*Vaccinium myrtillus* L.) growing at different locations. Journal of the Science of Food and Agriculture 95(4): 776-785.

Mitsuyama, K., Toyonaga, A. and Sata, M. 2005. Immunoregulatory cytokines and intestinal inflammation. The Journal of Gastroenterology 40(2): 217-219.

Murch, S., Lamkin, V., Savage, M., Walker-Smith, J. and MacDonald, T. 1991. Serum concentrations of tumour necrosis factor-alpha in childhood chronic inflammatory bowel disease. Gut 32(8): 913-917.

Ou, B., Hampsch-Woodill, M. and Prior, R. L. 2001. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. Journal of Agricultural and Food Chemistry 49(10): 4619-4626.

Pan, M.-H., Lai, C.-S. and Ho, C.-T. 2010. Anti-inflammatory activity of natural dietary flavonoids. Food and Function 1(1): 15-31.

Parveen, U. B., Roy, S. and Kumar, A. 2007. Traditional uses of medicinal plants among the rural communities of Churu district in the Thar Desert, India. Journal of Ethnopharmacology 113(3): 387-399.

Pearl, D. S., Shah, K., Whittaker, M. A., Nitch-Smith, H., Brown, J. F., Shute, J. K. and Trebble, T. M. 2013. Cytokine mucosal expression in ulcerative colitis, the relationship between cytokine release and disease activity. Journal of Crohn's and Colitis 7(6): 481-489.

Reinecker, H.-C., Loh, E. Y., Ringler, D. J., Mehta, A., Rombeau, J. L. and MacDermott, R. P. 1995. Monocyte-chemoattractant protein 1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. Gastroenterology 108(1): 40-50.

Reinecker, H., Steffen, M., Witthoeft, T., Pflueger, I., Schreiber, S., MacDermott, R. and Raedler, A. 1993. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. Clinical and Experimental Immunology 94(1): 174-181.

Rezaie, A., Parker, R. and Abdollahi, M. 2007. Oxidative stress and pathogenesis of inflammatory bowel disease: An epiphenomenon or the cause? Digestive Diseases and Sciences 52(9): 2015-2021.

Ribeiro, D., Freitas, M., Lima, J. L. F. C. and Fernandes, E. 2015. Proinflammatory pathways: The modulation by flavonoids. Medicinal Research Reviews 35(5): 877-936.

Rostom, A., Dubé, C., Lewin, G., Tsertsvadze, A., Barrowman, N., Code, C., Sampson, M. and Moher, D. 2007. Nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors for primary prevention of colorectal cancer: A systematic review prepared for the U.S. preventive services task force. Annals of Internal Medicine 146(5): 376-389.

Rutgeerts, P., Sandborn, W. J., Feagan, B. G., Reinisch, W., Olson, A., Johanns, J., Travers, S., Rachmilewitz, D., Hanauer, S. B., Lichtenstein, G. R., de Villiers, W. J. S., Present, D., Sands, B. E. and Colombel, J. F. 2005. Infliximab for induction and maintenance therapy for ulcerative colitis. New England Journal of Medicine 353(23): 2462-2476.

Sangiovanni, E., Di Lorenzo, C., Colombo, E., Colombo, F., Fumagalli, M., Frigerio, G., Restani, P. and Dell'Agli, M. 2015. The effect of *in vitro* gastrointestinal digestion on the anti-inflammatory activity of *Vitis vinifera* L. leaves. Food and Function

6(8): 2453-2463.

Shin, H. S., Satsu, H., Bae, M.-J., Zhao, Z., Ogiwara, H., Totsuka, M. and Shimizu, M. 2015. Anti-inflammatory effect of chlorogenic acid on the IL-8 production in Caco-2 cells and the dextran sulphate sodium-induced colitis symptoms in C57BL/6 mice. Food Chemistry 168: 167-175.

Singer, I., Kawka, D., Schloemann, S., Tessner, T., Riehl, T. and Stenson, W. 1998. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. Gastroenterology 115(2): 297-306.

Sonier, B., Patrick, C., Ajjikuttira, P. and Scott, F. W. 2009. Intestinal immune regulation as a potential diet-modifiable feature of gut inflammation and autoimmunity. International Reviews of Immunology 28(6): 414-445.

Sun, A., Xu, X., Lin, J., Cui, X. and Xu, R. 2015. Neuroprotection by saponins. Phytotherapy Research 29(2): 187-200.

Taverniti, V., Fracassetti, D., Del, B. C., Lanti, C., Minuzzo, M., Klimis-Zacas, D., Riso, P. and Guglielmetti, S. 2014. Immunomodulatory effect of a wild blueberry anthocyanin-rich extract in human Caco-2 intestinal cells. Journal of Agricultural and Food Chemistry 62(33): 8346-8351.

Tuntipopipat, S., Muangnoi, C., Chingsuwanrote, P., Parengam, M., Chantravisut, P., Charoenkiatkul, S. and Svasti, S. 2011. Anti-inflammatory activities of red curry paste extract on lipopolysaccharide-activated murine macrophage cell line. Nutrition 27(4): 479-487.

Tuntipopipat, S., Muangnoi, C., Thiyajai, P., Srichamnong, W., Charoenkiatkul, S. and Praengam, K. 2015. A bioaccessible fraction of parboiled germinated brown rice exhibits a higher anti-inflammatory activity than that of brown rice. Food and Function 6(5): 1480-1488.

Umehara, Y., Kudo, M., Nakaoka, R., Kawasaki, T. and Shiomi, M. 2006. Serum proinflammatory cytokines and adhesion molecules in ulcerative colitis. Hepatogastroenterology 53(72): 879-882.

Ursini, F., Maiorin, M. and Gregolin, C. 1985. The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. Biochimica et Biophysica Acta 839(1): 62-70.

Vichai, V. and Kirtikara, K. 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nature Protocols 1(3): 1112-1116.

Wang, D., DuBois, R. N. and Richmond, A. 2009. The role of chemokines in intestinal inflammation and cancer. Current Opinion in Pharmacology 9(6): 688-696.

Wolfe, K. L. and Rui, H. L. 2007. Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. Journal of Agricultural and Food Chemistry 55(22): 8896-8907.

Zhou, E., Liu, H., Wu, H., Shi, Z., Zhang, W., Zhu, Y., Shi, D. and Zhou, S. 2009. Down-regulation of protein and mRNA expression of IL-8 and ICAM-1 in colon tissue of ulcerative colitis patients by partition-herb moxibustion. Digestive Diseases and Sciences 54(10): 2198-2206.

Zhou, Z., Dai, C. and Liu, W. 2015. Anti-TNF-A therapy about infliximab and adalimamab for the effectiveness in ulcerative colitis compared with conventional therapy: a meta-analysis. Hepatogastroenterology 62(138): 309-318.

Zhu, H. and Li, Y. R. 2012. Oxidative stress and redox signaling mechanisms of inflammatory bowel disease: updated experimental and clinical evidence. Experimental Biology and Medicine 237(5): 474-480.