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In vitro binding of bile acids by spinach, kale, brussels sprouts, broccoli, mustard greens, green bell pepper, cabbage and collards ☆

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Abstract

The in vitro binding of bile acids by spinach (*Spinacia oleracea*), kale (*Brassica oleracea acephala*), Brussels sprouts (*Brassica oleracea gemmifera*), broccoli (*Brassica oleracea italica*), mustard greens (*Brassica juncea*), green bell peppers (*Capsicum annuum*), cabbage (*Brassica oleracea capitala*) and collards (*Brassica oleracea acephala*) was determined using a mixture of bile acids secreted in human bile at a duodenal physiological pH of 6.3. Six treatments and two blank incubations were conducted testing various fresh raw green vegetables on an equal dry matter basis. Considering cholestyramine (bile acid binding, cholesterol lowering drug) as 100% bound, the relative in vitro bile acid binding of various vegetables tested on equal dry matter and total dietary fibre basis was 2–9% and 6–32%, respectively. Bile acid binding for spinach, kale and brussels sprouts was significantly higher than for broccoli and mustard greens. For broccoli and mustard greens binding values were significantly higher those for cabbage, bell pepper and collards. These results point to the health promoting potential of spinach = kale = brussels sprouts > broccoli = mustard greens > cabbage = green bell peppers = collards, as indicated by their bile acid binding on dry matter basis.

Keywords: Spinach; Kale; Brussels sprouts; Broccoli; Mustard greens; Green bell pepper; Cabbage; Collards; Bile acid binding

1. Introduction

Vegetarians or those consuming vegetables as a major portion of their daily diet, along with consuming fewer calories from saturated fat and animal products are at a lower risk of coronary heart disease and cancer (Bazzano et al., 2002; Burr and Sweetnam, 1982; Chang-Claude et al., 1992; Fraser et al., 1981; Gaziano et al., 1995; Johnsen et al., 2003; Joshipura et al., 1999, 2001; Key et al., 1998, 1999a, 1999b, 2003; Law and Morris, 1998; Liu et al., 2000, 2001, 2003; Ness and Powles, 1997; Rissanen et al., 2003). USDA Food and Nutrition Information Center (2005) Food Guide Pyramid – Steps to a Healthier You (http://www.mypyramid.gov) recommends consumption of dark leafy and colourful vegetables. Some of the cruciferous vegetables recommended by the USDA food pyramid include broccoli, collard greens, kale and spinach. Isothiocyanates of the cruciferous vegetables of the brassica family have been shown to protect against various types of cancers (Fahey, Zhang, & Talalay, 1997; Steinmetz & Potter, 1990; Zhang, Talalay, Cho, & Posner, 1992, 1994). Sulforaphane, indole-3-carbinol, glucaric acid and other isothiocyanates are antioxidants and potent stimulators of natural detoxifying enzymes in the body. These compounds are believed to be responsible for the lowered risk of atherosclerosis and cancer (Ames, Shigenaga, & Hagen, 1993; Hecht, 1999). Toxic metabolites in the gut and secondary bile acids increase the risk of colorectal cancer (Costarelli et al., 2002). The healthful, cholesterol-lowering (atherosclerosis amelioration) or detoxification of harmful metabolites (cancer prevention) potential of food fractions could be predicted by evaluating their in vitro bile

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acid binding, based on positive correlations found between in vitro and in vivo studies showing that cholestyramine (bile acid binding, cholesterol lowering drug) binds bile acids and cellulose does not (Daggy, O'Connell, Jerdack, Stinson, & Setchell, 1997; Kahlon & Chow, 2000; Nakamura & Matsuzawa, 1994; Suckling et al., 1991). Bile acids are acidic steroids synthesized in the liver from cholesterol. After conjugation with glycine or taurine, they are secreted into the duodenum. Bile acids are actively reabsorbed by the terminal ileum and undergo an enterohepatic circulation (Hofmann, 1977). Binding of bile acids and increasing their faecal excretion has been hypothesized as a possible mechanism by which dietary fibre lowers cholesterol (Anderson & Siesel, 1990; Lund, Gee, Brown, Wood, & Johnson, 1989; Trowell, 1975). By binding bile acids, food fractions prevent their reabsorption and stimulate plasma and liver cholesterol conversion to additional bile acids (Balmer & Zilversmit, 1974; Eastwood & Hamilton, 1968; Kritchevsky & Story, 1974; Potter, 1998). Excretion of toxic metabolites and secondary bile acids could lower the risk of cancer (Costarelli et al., 2002). Bile acid binding of grain fractions as well as various dry beans has been observed to be proportional to their dry matter content (Kahlon & Woodruff, 2003a, 2003b; Kahlon & Shao, 2004; Kahlon, Smith, & Shao, 2005).

The objective of this study was to determine the healthful potential of spinach (*Spinacia oleracea*), kale (*Brassica oleracea acephala)* USDA nutrient data base number, NDN 11223], brussels sprouts (*Brassica oleracea gemmifera*), broccoli (*Brassica oleracea italica*), mustard greens (*Brassica juncea*), green bell peppers (*Capsicum annuum*), cabbage (*Brassica oleracea capitata*) and collards (*Brassica oleracea acephala*, NDN 11161) by evaluating their in vitro bile acid binding, using fresh green raw vegetables on an equal dry matter basis, with a bile acid mixture observed in human bile under duodenal physiological pH of 6.3.

2. Materials and methods

Fresh spinach, kale, brussels sprouts, broccoli, mustard greens, green bell pepper, cabbage and collards were obtained from a local super market. All the fresh vegetables were washed and lyophilized in a Lyph-lock 18 freeze dryer (Laconco Corporation, Kansas City, MO). Freeze dried samples were ground frozen using dry ice in a Thomas–Wiley mini mill (Arthur Thomas, Philadelphia, PA) to pass through a 0.4 mm screen. Samples were analyzed for total dietary fibre by method 991.16 (AOAC, 2005), nitrogen by method 990.03 (AOAC, 1990) with Vario Macro Elemental Analyzer (Elementar Analysensysteme GmbH, Hanau, Germany), crude fat with petroleum ether by the accelerated solvent extractor (ASE 200 Dionex Corp., Sunnyvale, CA), as described by Berrios, Camara, Torija, and Alonso (2002), ash by method 942.05 (AOAC, 1990) and for moisture by method 935.29 (AOAC, 1990). Cellulose, a non-bile acid binding fibre, was the negative control and cholestyramine, a bile acid binding anionic resin (a drug that lowers cholesterol and binding bile acids), was the positive control and both were obtained from Sigma (St. Louis, MO). Eight replicate incubations, six with bile acid mixture, one substrate blank without bile acid mixture and one bile acid mixture without substrate, were run for each treatment and control. All the vegetables used for incubation on dry matter basis were 99–104 mg and cellulose 24 mg and cholestyramine 26 mg.

2.1. Bile acid binding procedure

The in vitro bile acid binding procedure was a modification of that by Camire, Zhao, and Violette (1993), as previously reported (Kahlon & Chow, 2000). The stock bile acid mixture was formulated with glycocholic bile acids providing 75% and taurine-conjugated bile acids 25% of the bile acids, based on the composition of the human bile (Carey & Small, 1970; Rossi, Converse, & Hoffman, 1987). This mixture contained glycocholic acid (9 mmol/l), glycochenocholic acid (9 mmol/l), glycodeoxycholic acid (9 mmol/l), taurocholic acid (3 mmol/l), taurochenocholic acid (3 mmol/l) and taurodeoxycholic acid (3 mmol/l) in pH 6.3, 0.1 M phosphate buffer. This stock solution of 36 mmol/l was stored in the −20 °C freezer and diluted to the working solution (0.72 μmol/ml) just prior to each assay. Six replicates of 99–104 mg dry matter of spinach, kale, brussels sprouts, broccoli, mustard greens, green bell pepper, cabbage, collards, and cholestyramine (26 mg) and cellulose (24 mg) were tested. One substrate blank, one positive blank (2.88 μmol bile acid mixture per incubation) and six treatment replicates were weighed into 16 × 150 mm glass, screw-capped tubes. Samples were digested in 1 ml 0.01 N HCl for one hour in a 37 °C shaker bath. After this acidic incubation which simulated gastric digestion, the sample pH was adjusted to 6.3 with 0.1 ml of 0.1 N NaOH. To each test sample was added 4 ml of bile acid mixture working solution (0.72 μmol/ml) in a 0.1 M phosphate buffer, pH 6.3. A phosphate buffer (4 ml, 0.1 M, pH 6.3) was added to the individual substrate blanks. After the addition of 5 ml of porcine pancreatin (5×, 10 mg/ml, in a 0.1 M phosphate buffer, pH 6.3; providing amylase, protease and lipase for digestion of samples), tubes were incubated for one hour in a 37 °C shaker bath. Mixtures were transferred to 10 ml centrifuge tubes (Oak Ridge 3118-0010 Nalgene, Rochester, NY) and centrifuged at 99,000*g* for 18 min at 25 °C in an ultracentrifuge. Incubation tubes were rinsed with 5 ml phosphate buffer and centrifuged as before. Aliquots of pooled supernatant were frozen at −20 °C for bile acids analysis. Bile acids were analyzed using Trinity Biotech bile acids procedure No. 450 (Trinity Biotech Distribution, St. Louis, MO) using a Ciba-Corning Express Plus analyzer (Polestar Labs, Inc., Escondido, CA). Each sample was analyzed in triplicate. Values were determined from a standard curve obtained by analyzing Trinity Biotech bile acid calibrators (No. 450–11) at 5, 25, 50, 100 and 200 μmol/l. Individual substrate blanks were subtracted, and bile acid concentrations were corrected, based on the mean recoveries of bile acid mixture (positive blank). The effect of treatment was tested using Lavene's test for homogeneity and least square means were calculated. Dunnett's one-tailed test was used for comparison of cholestyramine and cellulose against all treatments, and differences among spinach, kale, brussels sprouts, broccoli, mustard greens, bell pepper, cabbage and collards were tested for significance with Tukey's test for comparison of all possible pairs of means (SAS Institute, Cary, NC). A value of $P \leqslant 0.05$ was considered the criterion of significance.

3. Results and discussion

Composition of the spinach, kale, brussels sprouts, broccoli, mustard greens, green bell pepper, cabbage and collards is given in Table 1. Both cellulose and cholestyramine were considered as 100% total dietary fibre. There was wide variation in the total dietary fibre (27–36%), protein (17–41%), fat (4–12%) and ash (10–28%) content of these vegetables. Carbohydrate (29–69%) content was determined by difference, Carbohydrates = [100 − (protein + crude fat + ash)].

On an equal dry matter (DM) basis, bile acid binding was significantly higher for cholestyramine than all the fresh green vegetables tested (Table 2). Spinach, kale and brussels sprouts bound bile acids similarly and significantly ($P \leqslant 0.05$) higher than broccoli, mustard greens, green bell pepper, cabbage and collards. Bile acid binding values for broccoli and mustard greens were also similar and significantly higher than green bell pepper, cabbage collards and cellulose. Cholestyramine bound 92% of the bile acids. These values are similar to the previously reported observations (Kahlon & Chow, 2000). Cholestyramine bound glycocholate and taurocholate 87% and 93%, respectively (Sugano & Goto, 1990). In our study cholestyramine binding to the mixture of bile acids was similar to that observed for taurocholate by Sugano and Goto (1990). Story and Kritchevsky (1976) reported 81% bile acid binding by cholestyramine using 50 mg of substrate and 50 μmol of bile acids. Higher bile acid binding by cholestyramine in our studies may be due to the use of physiological pH and/or a higher substrate to bile acid ratio.

Table 1
Composition of spinach (*Spinacia oleracea*), kale (*Brassica oleracea acephala*), brussels sprouts (*Brassica oleracea gemmifera*), broccoli (*Brassica oleracea italica*), mustard greens (*Brassica juncea*), green bell pepper (*Capsicum annuum*), cabbage (*Brassica oleracea capitala*) and collards (*Brassica oleracea acephala*), dry matter (DM) basis

Source	DM (%)				
	Total dietary fibre[a]	Protein[b]	Fat[b]	Ash[b]	Carbohydrate[c]
Spinach	27.1	38.2	4.6	28.1	29.0
Kale	36.8	33.8	11.8	15.8	38.6
Brussels sprouts	34.1	29.0	4.1	9.5	57.4
Broccoli	31.1	33.2	5.4	10.3	51.2
Mustard greens	34.0	40.9	3.9	16.7	38.5
Green bell pepper	26.5	16.9	3.7	10.5	68.9
Cabbage	29.9	23.1	4.4	10.5	62.0
Collards	36.3	21.3	5.8	16.3	56.6
Cholestyramine	100.0	–	–	–	–
Cellulose	100.0	–	–	–	–

[a] $n = 6$.
[b] $n = 3$.
[c] Carbohydrates = [100 − (protein + crude fat + ash)].

Table 2
In vitro bile acid binding by spinach (*Spinacia oleracea*), kale (*Brassica oleracea acephala*), brussels sprouts (*Brassica oleracea gemmifera*), broccoli (*Brassica oleracea italica*), mustard greens (*Brassica juncea*), green bell pepper (*Capsicum annuum*), cabbage (*Brassica oleracea capitala*) and collards (*Brassica oleracea acephala*) on equal weight, dry matter (DM) basis[A,B]

Treatment	Bile acid binding (μmol/100 mg DM)	Bile acid binding relative to cholestyramine, %
Spinach	0.87 ± 0.04^{b}	8.6 ± 0.4^{b}
Kale	0.83 ± 0.04^{b}	8.2 ± 0.4^{b}
Brussels sprouts	0.79 ± 0.02^{b}	7.8 ± 0.2^{b}
Broccoli	0.47 ± 0.04^{c}	4.6 ± 0.4^{c}
Mustard greens	0.44 ± 0.01^{c}	4.3 ± 0.4^{c}
Green bell pepper	0.25 ± 0.01^{d}	2.5 ± 0.1^{d}
Cabbage	0.24 ± 0.01^{d}	2.4 ± 0.1^{d}
Collards	0.21 ± 0.02^{d}	2.1 ± 0.2^{d}
Cholestyramine	10.14 ± 0.09^{a}	100.0 ± 0.9^{a}
Cellulose	0.16 ± 0.05^{d}	1.6 ± 0.5^{d}

[A] Mean ± SEM within a column with different superscript letters differ significantly ($P \leqslant 0.05$), $n = 6$.
[B] The dry matter used for incubation was all the vegetables was 99–104 mg, cholestyramine and cellulose 24–26 mg.

Assigning a bile acid binding value of 100% to cholestyramine, the relative bile acid binding on dry matter basis for the test samples of fresh green vegetables was spinach 9%, kale 8%, brussels sprouts 8%, broccoli 5%, mustard greens 4%, green bell pepper 3%, cabbage 2% and collards 2%. Bile acid binding for spinach, kale and brussels sprouts was similar (8–9%) and significantly higher than broccoli, mustard greens, green bell pepper, cabbage and collards. Bile acid binding for broccoli and mustard greens was similar (4–5%) and significantly higher than green bell pepper, cabbage and collards (2–3%). Relative bile acid binding on dry matter basis was spinach = kale = brussels sprouts > broccoli = mustard greens > green bell pepper = cabbage = collards.

On a dry matter basis bile acid binding of spinach, kale and brussels sprouts is very encouraging, and could indicate their significantly higher healthful potential than broccoli, mustard greens, green bell pepper, cabbage and collards. Bile acid binding on a dry matter basis has been reported as a measure of relative healthful potential of ready to eat cereals, cereal fractions and dried beans (Kahlon & Woodruff, 2003a, 2003b; Kahlon & Shao, 2004; Kahlon et al., 2005). Binding bile acids and preventing their recirculation results in reduced fat absorption, excretion of cancer-causing toxic metabolites and cholesterol utilization to synthesize more bile acids. This is believed to be the mechanism by which food fractions lower cholesterol and prevent cancer. Evaluating the healthful properties of various vegetables and food fractions would be desirable by testing their bile acid binding on a dry matter basis.

Kale (*Brassica oleracea, acephala* NDN 11223), brussels sprouts (*Brassica oleracea gemmifera*), broccoli (*Brassica oleracea italica*), mustard greens (*Brassica juncea*), cabbage (*Brassica oleracea capitata*) and collards *(Brassica oleracea acephala* NDN 11161), are all green vegetables of the *Brassica* family and their bile acid binding potential on dry matter basis is quite varied. Kale (*acephala*), brussels sprouts (*gemmifera*), broccoli (*italica*) and cabbage (*capitala*) are four varieties of *Brassica oleracea*. In addition kale NDN 11223 and collards NDN 11161 are two stains of the *acephala* variety of the *Brassica oleracea*. The differences in bile acid binding between various varieties and strains of *brassica* vegetables may relate to their phytonutrients (isothiocyanates, flavonoids, chlorophyll, tannins, micro elements), hydrophobicity or active binding sites. Isothiocyanates of the cruciferous vegetables of the brassica family have been shown to protect against various types of cancers (Steinmetz & Potter, 1990; Zhang et al., 1992, Zhang, Kensler, & Cho, 1994). Sulforaphane and other isothiocyanates are antioxidants and potent stimulators of natural detoxifying enzymes in the body. These compounds are believed to be responsible for a lowered risk of atherosclerosis and cancer (Ames et al., 1993; Hecht, 1999).

The bile acid binding on equal total dietary fibre (TDF) basis is shown in Table 3. Cholestyramine bound bile acids significantly higher and cellulose significantly lower than the various cruciferous green vegetables tested. On a TDF basis considering cholestyramine as 100% bound, bile acid binding values were spinach 32%, kale 22%, brussels sprouts 23%, broccoli 15%, mustard greens 13%, green bell pepper 9%, cabbage 8% and collards 6%. The bile acid binding for spinach was significantly higher than for all the other vegetables tested. Bile acid binding values for broccoli and mustard greens were significantly lower than those for kale and Brussels sprouts, but significantly higher than those of green bell pepper, cabbage and collards. Bile acid binding values on TDF basis among various green vegetables tested were spinach > kale = brussels sprouts > broccoli = mustard greens > green bell pepper = cabbage = collards. Over fivefold variability in the bile acid binding among various green vegetables tested indicated that bile acid binding is not related to the TDF content. This is in agreement with previous reports that bile acid binding of black eye bean, garbanzo, kidney bean, lima bean, moth bean and soybean was not related to the TDF content (Kahlon & Shao, 2004; Kahlon et al., 2005). The variability in bile acid binding between the green vegetables tested may be related to their phytonutrients, isothiocyanates, sulforaphane, flavonoids, tannins, anionic, cationic, physical and chemical structure.

Table 3
In vitro bile acid binding by spinach (*Spinacia oleracea*), kale (*Brassica oleracea acephala*), brussels sprouts (*Brassica oleracea gemmifera*), broccoli (*Brassica oleracea italica*), mustard greens (*Brassica juncea*), green bell pepper (*Capsicum annuum*), cabbage (*Brassica oleracea capitala*) and collards (*Brassica oleracea acephala*) on equal total dietary fibre (TDF) basis[A,B]

Treatment	Bile acid binding (μmol/100 mg TDF)	Bile acid binding relative to cholestyramine (%)
Spinach	3.23 ± 0.14^{b}	31.9 ± 1.4^{b}
Kale	2.27 ± 0.10^{c}	22.4 ± 1.0^{c}
Brussels sprouts	2.32 ± 0.05^{c}	22.9 ± 0.5^{c}
Broccoli	1.50 ± 0.14^{d}	14.8 ± 1.4^{d}
Mustard greens	1.28 ± 0.04^{d}	12.6 ± 0.4^{d}
Green bell pepper	0.95 ± 0.04^{e}	9.4 ± 0.4^{e}
Cabbage	0.81 ± 0.04^{e}	7.9 ± 0.4^{e}
Collards	0.57 ± 0.05^{e}	5.7 ± 0.4^{e}
Cholestyramine	10.14 ± 0.09^{a}	100.0 ± 0.9^{a}
Cellulose	0.16 ± 0.05^{f}	1.6 ± 0.5^{f}

[A] Mean ± SEM within a column with different superscript letters differ significantly ($P \leqslant 0.05$), $n = 6$.
[B] The TDF (mg) used for incubation was spinach 27 mg, kale 37 mg, brussels sprouts 34 mg, broccoli 31 mg, mustard greens 34 mg, green bell pepper 26 mg, cabbage 30 mg, collards 36 mg, cholestyramine 26 mg and cellulose 24 mg.

The chemical composition of TDF of various vegetables tested was not found in the literature search nor was it determined; therefore it is not possible to speculate that variability in binding bile acids may relate to the differences in polysaccharides present in their dietary fibre fractions. Raw fresh vegetables are believed to be more nutritious and are consumed in salads, sandwiches and/or with dips. However cooking and/or cooking methods may alter binding sites or fibre molecules; such effects would be explored to evaluate healthful potential of various vegetables as generally consumed by humans.

In conclusion, relative to cholestyramine, in vitro bile acid binding on an equal DM and TDF basis were spinach 9% and 32%, kale 8% and 22%, brussels sprouts 8% and 23%, broccoli 5% and 15%, mustard greens 4% and 13%, green bell pepper 3% and 9%, cabbage 2% and 8%, and collards 2% and 6%. On dry matter basis bile acid binding was spinach = kale = brussels sprouts > broccoli = mustard greens > green bell pepper = cabbage = collards. Relative bile acid binding on DM basis may indicate their relative health promoting potential. The difference in bile acid binding between various green vegetables tested may relate to the variability in their phytonutrients (isothiocyanates, sulforaphane, indole-3-carbinol, glucaric acid, flavonoids, tannin, micro-elements), non-protein composition, structure, hydrophobicity of undigested fractions, anionic or cationic nature of the metabolites produced during digestion or their interaction with active binding sites. Studies are planned to evaluate how cooking methods impact the bile acid binding of vegetables and animal studies to validate in vitro bile acid binding of vegetables observed herein to their healthful potential of atherosclerosis amelioration (lipid and lipoprotein lowering) and cancer prevention (excretion of toxic metabolites).

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