UNDERSTANDING THE RELATIONSHIP OF BILE ACID BINDING CAPACITY, PHENOLIC COMPOUNDS AND THEIR BIOACCESSIBILITY OF SELECTED VEGETABLES

A Thesis

by

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ABSTRACT

Vegetables with bile acid capacity provide multiple benefits to human health, including reducing plasma cholesterol levels, controlling blood sugar in type 2 diabetic patients, and preventing colon cancer. The *in vitro* bile acid binding capacities of Brussels sprouts, green kale, red kale, red cabbage, and red leaf lettuce were tested and their dietary fiber content was analyzed. Green kale was used for further studies to explore the optimal dose for bile acid binding, the effect of bile acid composition on the binding capacity, the influence of minimal processing on the bile acid binding capacity, the interaction of bioactive compounds with bile acids, and the impact of bile acids on the bioaccessibility of kale polyphenols.

The *in vitro* digestion was conducted in three phases that simulated oral, gastric, and intestinal digestion. Bile acids were incubated with vegetables in the intestinal digestion phase. Kale had the greater bile acid binding capacity compared with Brussels sprouts, red cabbage, and red leaf lettuce. In the experiment testing the effect of different bile acid compositions on bile acid binding capacity, kale showed a similar binding capacity for the bile acid composition simulating that found in healthy females and males with gallstones, but it bound less bile acids when the composition simulated the bile acid pool of type-2 diabetic males. The type-2 diabetic male patient model was used to explore bile acid binding capacity in response to different doses of kale. The results suggested that the optimal dose of kale was 1.8 g, which bound 81.8% of the added bile acid. Microwaving and steaming significantly improved kale's *in vitro* bile acid binding capacity.

To study the interaction between bile acids and different bioactive compounds in kale, polyphenols were separated from the fiber-rich kale tissue, and both of these were incubated

with bile acids. We found that the fiber-rich tissue in kale was the main component that binds bile acids. Similar *in vitro* digestions both with and without bile acids suggested that bile acids improved the bioaccessibility of quercetin and the total identified polyphenols in kale. Therefore, bile acids can be bound by fiber rich tissues in the kale and have some interactions with kale polyphenols.

DEDICATION

To my loving and courageous mom, my true friends, and supportive professors.

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NOMENCLATURE

BA	Bile acid
GCA	Glycocholate
CA	Cholate
GCDCA	Glycochenodeoxycholate
GDCA	Glycodeoxycholate
CDCA	Chenodeoxycholate
DCA	Deoxycholate
BAC	Bile acid composition

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CHAPTER I

INTRODUCTION

Bile acids are emulsifiers that facilitate the digestion of fat. These amphipathic molecules have a hydrophilic side and a hydrophobic side and can surround fat globules in the aqueous environment to form a lipid drop. Lipases can then attach to the lipid drop and assist with fat digestion. Bile acids are synthesized from cholesterol in the liver, stored in the gallbladder, and enter the small intestine when fatty food is in the digestive system. The human body has fourteen bile acids: two of them are primary bile acids, namely cholic acid, chenodeoxycholic acid,[1] and two of them are secondary bile acids, namely deoxycholic acid and lithocholic acid. Each of these four bile acids can attach to taurine or glycine in the liver and form conjugated bile acids. Our bodies also have a trace amount of ursodeoxycholic acid.[2] Unconjugated bile acids are more hydrophobic than conjugated bile acids, which are more toxic to liver and colon cells.[3] Deoxycholic acid and lithocholic acid can even promote colon cancer.[4] Therefore, binding bile acids through foods can protect the colon and liver and also promote the synthesis of new bile acids from cholesterol, which consequently reduces plasma cholesterol levels.

Various vegetables have *in vitro* bile acid binding capacity, including beets, collard greens, kale, mustard greens, broccoli, Brussels sprouts, and spinach.[5] These vegetables are rich in dietary fiber, which is commonly considered as the component that binds bile acids. Red cabbage, red kale, green kale, Brussels sprouts, and red leaf lettuce all contain a good amount of dietary fiber and other nutrients. Red cabbage and red leaf lettuce have glucosinolates and anthocyanins.[6] The glucosinolates in Brussels sprouts can lower the risk of cancer.[7] Kale has lutein, chlorophyll, glucosinolates, and polyphenols.[8-10] Among these

vegetables, green kale has a higher content of antioxidants, which is due to its abundant polyphenols.[11]

In addition to digestion emulsification, bile acids also assist in the absorption of some nutrients in vegetables, such as beta-carotene, lycopene, and lutein. When bile acids are absorbed through the ileum and pass into the blood stream, they carry along the liposoluble nutrients.[12] Yet, dietary fiber in the plant can bind bile acids and affect bile acid absorption. It can bind bile acids and potentially reduce cholesterol levels and protect the liver and the colon. However, not all bile acids are toxic to the liver or colon; some hydrophilic bile acids, such as tauroursodeoxycholic acid, have protective functions in the human body.[3] Therefore, it is necessary to study the binding capacities for hydrophilic and hydrophobic bile acids separately. Even though vegetables' *in vitro* bile acid binding capacity has been investigated before,[5, 13] few studies have examined the binding capacity of individual bile acids.

Bile acids can be bound by dietary fiber, and also by some polyphenols. For example, Ngamukote found grape polyphenols can bind bile acids and reduce cholesterol levels.[14] It is possible that the phenolic compounds in the vegetables may also contribute to *in vitro* bile acid binding capacity. On the other hand, bile acids may affect absorption of polyphenols in the digestive system because during digestion, dietary fiber can bind bile acids and can also trap some phenolic compounds.[12] Therefore, bile acids might influence the bioaccessibility of polyphenols, since they both can interact with dietary fiber. According to the best of our knowledge, the effect of bile acids on polyphenol bioaccessibility has not yet been studied.

This research was designed to study the *in vitro* binding capacity for total and individual bile acids by kale, Brussels sprouts, red leaf lettuce and red cabbage. The interaction of bile acids with dietary fiber and kale polyphenols was also explored.

CHAPTER II

LITERATURE REVIEW

Bile Acids

The human liver can synthesize primary bile acids — cholic acid and chenodeoxycholic acid — from cholesterol, and the bacteria in the intestine can transform primary bile acids into secondary bile acids, namely lithocholic acid and deoxycholic acid.[15] When these unconjugated bile acids bind to taurine and glycine, conjugated bile acids are formed, including taurocholic acid, taurodeoxycholic acid, taurochenodeoxycholic acid and taurolithocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid, and glycolithocholic acid. The bacteria in the human gastric intestinal tract can deconjugate the taurine-formed and glycine-formed bile acids and transfer them back into unconjugated bile acids.[16]

When the human digestive system breaks down fat, bile acids assist digestion by acting as emulsifiers in the intestine. During digestion, 95% of bile acids are reabsorbed through the ileum and transferred back to the liver; only 5% of bile acids are excreted out of the human body.[2] Because bile acids are reabsorbed, the human bile pool remains constant; therefore, effective bile acid recycling suppresses the synthesis of new bile acids from cholesterol. Meanwhile, the modern western diet has large amounts of lipids, which can contribute to the accumulation of cholesterol in the human body, and may develop into hypercholesterolemia or cardiovascular diseases if it is not controlled.[1, 2] Binding bile acids can contribute to the reduced cholesterol levels in blood and have protective function to hypercholesterolemia and cardiovascular disease.

Besides preventing hypercholesterolemia, binding bile acids can also lower the risk of colon cancer, since free bile acids in the colon irritate the colon cells and cause diarrhea, and hydrophobic bile acids, such as deoxycholic acid, chenodeoxycholic acid, can increase the potential of developing colon cancer.[17] In addition, Prawitt suggested that bile acids can activate a farnesoid X receptor (FXR), which is an inhibitor of hepatic glucose production and can regulate glucose metabolism.[18] Thus, binding bile acids can contribute to the control of blood sugar levels in type 2 diabetic patients.

People with malabsorption of bile acids may have too much free bile acid in their large intestine, which irritates the intestinal cells and causes diarrhea. And also, the bacteria in the intestine may release bound bile acids from the fiber, deconjugate the conjugated bile acid and form more secondary bile acids, which are more toxic to intestinal cells. In this case, bile acid sequestrant — cholestyramine, rather than fiber can help to protect intestinal cells from exposure to bile acids.[19] In addition, during the recycling of bile acids, some toxic compounds may attach to bile acids and be transferred to the liver together.[20] Binding bile acids can reduce the absorption of toxic compounds into the liver and have a detoxification function.

In summary, binding bile acids can potentially lower cholesterol levels, prevent colon cancer, protect the liver, and control bile acid diarrhea.

Selected Vegetables

Various vegetables have many health benefits, with anti-cancer, anti-aging, and anti-inflammatory effects. There is high amount of dietary fiber in red cabbage, red kale, Brussels sprouts, red leaf lettuce, and green kale, which helps to reduce plasma cholesterol

levels through binding bile acids.[5] There are glucosinolates, anthocyanins, and some polyphenols in red cabbage and red leaf lettuce, including hydroxycinnamic acids, chlorogenic acid, caffeic acid, and dicaffeoyltartaric acid.[6] Green kale and red kale have antioxidants, minerals, lutein, and vitamin C.[21] The glucosinolates in Brussels sprouts promote the activity of phase II enzymes, and can prevent cancer.[7] Among these vegetables, kale has become the most popular and has been embraced by consumers. It has the highest antioxidant activity (85.79%) in 1,1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging analysis, much higher than white cabbage, Chinese cabbage, and choy sum.[11] This suggests that kale can rapidly quench peroxides in the human body, which means kale can relieve oxidative stress before it causes damage to the human body. The main antioxidants in kale are kaempferol derivatives, quercetin derivatives, and some sinapoyl derivatives. For example, kale has kaempferol-3-O-sophoroside, kaempferol-3-(feruloyl)-sophoroside-7-diglucoside, quercetin-3-glucoside-7-O-glucoside, quercetin-3-sinapyl-diglucoside-7-diglucoside, disinapoyl-diglucoside, and sinapoyl-2-feruloyl-diglucoside. [22] Therefore, studying these polyphenols in kale can help us understand its potential health benefits.

Quercetin has an inhibitory effect on proliferation of lung cancer cells and colon cancer cells, and can induce apoptosis.[23, 24] Quercetin induces apoptosis in colon cancer cells by inhibiting the NF-κB pathway,[23] and it shows an antiproliferative effect on lung cancer by inhibiting the expression of the *Bcl-2* gene and promoting the expression of *Bax*, since Bcl-2 inhibits apoptosis and Bax induces apoptosis; in this way, quercetin increases the ratio of Bax/Bcl-2 protein, and consequently inhibit the growth of lung cancer cells.[24] Kaempferol, as a phytoestrogen, can supress ovarian cancer development by repressing the (ERK)-NF-Kb-cMyc-p21-VEG pathway.[25] It can also reduce breast cancer cell proliferation by regulating

the p53 tumor suppressor gene.[26] Kaempferol suppressed metastasis in osteosarcoma cells through interference with the ERK-p38-JNK and AP-1 signaling pathways.[27] In addition, kaempferol have a protective function against skin cancer by suppressing the expression of cyclooxygenase-2 (*COX-2*), which is induced by ultraviolet B light.[28] Sinapoyl derivatives have less antioxidant activity than sinapic acid, but still demonstrate high activity (IC_{50}=500 μM) in DPPH scavenging assays.[29]

Interaction Between Bile Acids and Dietary Fiber in the Vegetable

Since green kale is a good source for kaempferol, quercetin and dietary fiber. It contains 3.6% dietary fiber[21], which is higher than red cabbage, red leaf lettuce and Brussels sprouts. Kaempferol and quercetin derivatives in kale have outstanding antioxidant activity[11], it was chosen for the study of interactions with bile acids. Dietary fiber in kale is associated with health benefit of reducing plasma cholesterol contents. [30] Kahlon's research demonstrated that kale has good *in vitro* bile acid binding capacity.[5] These studies also found that steamed cooked kale has higher *in vitro* bile acid binding capacity than uncooked kale.[31] This could be because dietary fiber or proteins changed in structure during cooking. Kahlon compared the *in vitro* bile acid binding capacity and dietary fiber contents of different vegetables, including green collards, kale, okra, spinach, beets, mustard greens, broccoli, green bell peppers, cabbage, asparagus, eggplant, turnips, green beans, carrots, and cauliflower, and found no direct relationship between bile acid binding capacity and total dietary fiber content in vegetables.[13, 31] Hence it is possible that dietary fiber may not be the only substance in the vegetable that binds bile acids.

Interaction Between Bile Acids and Polyphenols in the Vegetable

Besides dietary fiber, bile acids also interact with other compounds. Lipid-soluble nutrients, such as carotene, lycopene, and lutein, require bile acids for absorption. People with a bile acid deficiency may have lower absorption of lipid-soluble nutrients, which would contribute to a deficiency of vitamin D.[19] Besides carotenoids and vitamin D, phenolic compounds in vegetables may also interact with bile acids. Han's research indicated that dietary polyphenols, curcumin, caffeic acid, rutin and ellagic acid, are able to decrease the toxic bile acids (deoxycholic and lithocholic acids) in feces, probably because the polyphenols can suppress the biosynthesis of bile acids from cholesterol together with cholesterol biosynthesis.[32] Kale has abundant kaempferol derivatives, quercetin derivatives, and sinapic acid derivatives,[33] and it is possible that these polyphenols may enhance the bile acid binding by dietary fibers in kale.

Even if polyphenols do not bind bile acids, bile acids in the digestive process may affect the releasing of polyphenols in the gastrointestinal tract. Nutrients that can release from the food matrix into the gastrointestinal tract are called bioaccessible nutrients. Bioaccessibility is a precondition of bioavailability. Bioavailable nutrients can be released from the food matrix during digestion, enter the gastric intestinal tract,[34] enter the bloodstream, reach a specific location in the human body, become available at the site of action, and have a physiological function. The main phenolic compounds in kale, kaempferol and quercetin, have excellent antioxidant capacity, which may slow down the aging process and suppress cancer. However, the bioavailability of kaempferol is very low, only 2%,[35] and the bioavailability of quercetin is 4%[36]. Yet, even though bioavailability of kaempferol and quercetin are low, they can still have a protective function against cancer. For instance,

antioxidants with higher bioavailability may not be helpful, but bioaccessible kaempferol and quercetin can fight against colon cancer. Because colon cancer cells occur in the gastrointestinal tract, cells exposed to these antioxidants may be affected and their proliferation inhibited. [23, 37] Therefore, studying the bioaccessibility of phytochemicals will provide practical information in cancer prevention. According to our best knowledge, little research has been done on the effect of bile acids on polyphenol bioaccessibility.

This research is designed to study the *in vitro* bile acid binding capacity of green kale, red cabbage, red leaf lettuce, red kale, and Brussels sprouts. Green kale was used to identify the optimum dose for *in vitro* bile acid binding. Also, green kale was processed by microwave cooking and steaming and its *in vitro* bile acid binding capacity after cooking was studied. The bioaccessibility of polyphenols in kale was also compared through *in vitro* digestions with and without bile acids.

CHAPTER III

IN VITRO BILE ACIDS BINDING CAPACITIES OF DIFFERENT VEGETABLES

Dietary fiber can bind bile acids thereby lowering the reabsorption of bile acids. This promotes the utilization of cholesterol to synthesize new bile acids, which can help to reduce plasma cholesterol levels. Secondary bile acids are also associated with colon cancer; therefore, reducing the free secondary bile acids can limit the risk of developing cancer. The present study describes the *in vitro* bile acid binding capacity of red leaf lettuce (*Lactuca sativa*) and the cruciferous vegetables red cabbage, red kale, green kale, and Brussels sprouts (*Brassica oleracea*). Green kale had the highest bile acid binding and we used this kale to determine the optimal dose to reach maximal bile acid binding capacity. The bound individual bile acids were quantified by reversed-phase HPLC. Further, we tested three different bile acid compositions that simulate various human health conditions (male with gallstones, healthy female, and male with type 2 diabetes). To predict the optimal dose for the best bile acid binding for each condition, we established the logistic relationship between different kale doses and bile acid binding capacities. The results indicated that kale has the highest bile acid binding capacity and it shows no significant difference in binding the bile acid compositions that mimic a healthy female and a male with gallstones, but it binds less bile acids from the profile of the type 2 diabetic male. Furthermore, the maximum amount (81.8%) of total bile acids was bound by green kale (1.8 g per 72.06 mg bile acids), and this remained the same up to 2.5 g. After understanding the dose-response using dried vegetables, we found that minimally processed (steamed) fresh kale bound even more bile acids. Among the six bile acids tested, kale preferentially bound the hydrophobic bile acids,

chenodeoxycholic acid and deoxycholic acid. Therefore, regular consumption of steam-cooked kale has the potential to improve human health by binding more bile acids.

Introduction

Bile acids are a group of amphipathic molecules that function as emulsifiers to assist in digestion and absorption of lipids in the gastrointestinal tract. Cholesterol can biosynthesize bile acids, which include cholic acid (CA), chenodeoxycholic acid, lithocholic acid (LCA), deoxycholic acid (DCA), glycochenodeoxycholic acid (GCDCA), glycocholic acid (GCA), and glycodeoxycholic acid (GDCA).[1] The two primary bile acids, cholic acid and chenodeoxycholic acid, are synthesized by the liver, stored in the gallbladder, and then secreted into the small intestine, when food is being digested.[2] Human intestinal bacteria can convert cholic acid and chenodeoxycholic acid to secondary bile acids such as deoxycholic acid and lithocholic acid though dehydroxylation. After assisting in lipid digestion, bile acids are reabsorbed through the ileum and transferred back to the liver. Up to 95% of bile acids are reabsorbed,[1, 2] which limits the synthesis of new bile acids from cholesterol. Some bile acids reach the colon, where gut bacteria form more deoxycholic acid and lithocholic acid.[1] Lithocholic acid, deoxycholic acid, and chenodeoxycholic acid are hydrophobic bile acids, which are considered more toxic to the liver and colon cells, and may promote colon cancer.[4, 38, 39] However, dietary fiber increases the excretion of bile acids in the stool,[40] which can potentially suppress colon cancer and lower the cholesterol level.[41] Hence, binding bile acids can reduce intestinal cancer and reduce plasma cholesterol levels. [42, 43]

When hydrophobic bile acids are conjugated with taurine or glycine, they become more hydrophilic and much less toxic to hepatic and intestinal cells.[44] Some conjugated bile acids,

such as tauroursodeoxycholic acid, can even stabilize hepatocyte membranes and enhance the defense against oxidative stress, thus protecting the liver.[3] Bile acid sequestrants prevent bile acid absorption and promote synthesis of primary bile acids from cholesterol, which leads to a reduction of serum cholesterol.[45] Some foods can function like bile acids sequestrants; for instance, guar gum can reduce the concentration of serum bile acids by binding them in the gastrointestinal tract, which relieves the symptoms associated with intrahepatic cholestasis of pregnancy,[46] and reduces plasma cholesterol levels.[47]

Beyond their functions of digestive emulsification and reduction of cholesterol, bile acids are also associated with glucose metabolism. Because bile acids can activate farnesoid X receptor and G-protein coupled bile acid receptor 1, which are involved in glucose metabolism, the control of bile acid absorption can be used as a new therapy for some metabolic diseases, such as type 2 diabetes.[2, 46] In fact, one of the bile acid sequestrants, colesevelam hydrochloride, was approved by the FDA in 2008 as a medicine for the treatment of type-2 diabetes.[48] In addition, bile acid binding has recently emerged as a supporting approach to strengthen the standard treatments to control plasma glucose in type-2 diabetic patients.[49] Several studies suggested that bile acids sequestrants reduced the fasting plasma glucose level in type-2 diabetes patients.[49-52] Therefore, a vegetable with good bile acid binding capacity may also lower the plasma glucose level of type-2 diabetic patients.

Previous studies have examined the bile acid binding capacities of various vegetables, but few studies have examined the binding preferences for individual bile acids by specific vegetables.[5] According to the previous research, collard greens, kale, mustard greens, broccoli, Brussels sprouts, spinach, green bell peppers, and cabbage have good bile acid binding capacity. [5] However, whether hydrophobic or hydrophilic bile acids are

preferentially bound by the vegetables has yet to be tested. Whether the different compositions of bile acid mixtures can influence the *in vitro* binding capacity also remains unknown. This study compared the *in vitro* bile acid binding capacities of different vegetables, including red cabbage, red leaf lettuce, Brussels sprouts, red kale, and green kale. The most active vegetable was used to test different bile acid compositions and optimal doses for bile acid binding.

Materials and Methods

Materials

Fresh green kale, red cabbage, red kale, Brussels sprouts, and red leaf lettuce were purchased from a local market. All vegetables were cut into small pieces, lyophilized and powdered prior to use in this study.

Chemicals

Sodium glycocholate, sodium cholate, sodium glycochenodeoxycholate, sodium glycodeoxycholate, sodium chenodeoxycholate, sodium deoxycholate, ammonium nitrate, potassium dihydrogen phosphate, potassium chloride, potassium citrate, uric acid sodium salt, urea, lactic acid sodium salt, porcine gastric mucin, α-amylase, pepsin, and pancreatin were obtained from Sigma-Aldrich Co (St. Louis, MO, USA).

Screening of Different Vegetables for Bile Acid Binding Ability

The *in vitro* digestion was performed to mimic digestion in the mouth, stomach, and small intestine (Fig. 1). A simulated saliva fluid (SSF) was prepared by dissolving the

reagents (Table S1) in pH 6.8 phosphate buffer.[53, 54] In the oral digestion phase, 2.0 g lyophilized vegetable was mixed with an equal weight of water and 10 ml of the SSF containing 0.31 mg α-amylase. The samples were vortexed and then placed in a shaking water bath (37°C and 180 rpm) for 5 mins. During the gastric digestion phase, the mixture from the oral digestion phase was adjusted to pH 2.0 with 1 N HCl, followed by the addition of 600 µl pepsin buffer (200 µg pepsin in 1 ml 0.1 M HCl) vortexing and incubation in a shaking water bath (37°C and 180 rpm) for 1 hour. For the intestinal phase, chyme from the gastric digestion phase was first adjusted to pH 6.8 with 1 N NaOH. The sample was then mixed with 5 ml pancreatin (6.25 mg/ml in 50 mM phosphate buffer), 4 ml bile acid mixture solution prior to incubation in a shaking water bath (37°C and 180 rpm) for 3 h.[53-56] The bile acid mixture contained 10 mM cholate, 10 mM deoxycholate, 10 mM glycochenodeoxycholate, 10 mM glycocholate, and 10 mM chenodeoxycholate in potassium phosphate buffer (pH 6.8). After incubation with the bile acids, the *in vitro* digestion was terminated by inactivating enzymes in a 78°C water bath for 7 min. The analyses were conducted using triplicate samples of freeze dried red cabbage, red kale, green kale, red leaf lettuce, and Brussels sprouts (2.0 g each). Digestion chymes were centrifuged at 3600 rpm and supernatants were collected and filtered through Whatman 90 mm filter paper. The residue was washed with 20 ml of nanopure water by mixing on a shaking water bath for 3 h and centrifuged at 800 g. Both the supernatants were filtered, combined, and concentrated under vacuum by rotary evaporation at 40°C to obtain 7 ml, and passed through 0.45 µm cellulose filters for HPLC analysis.

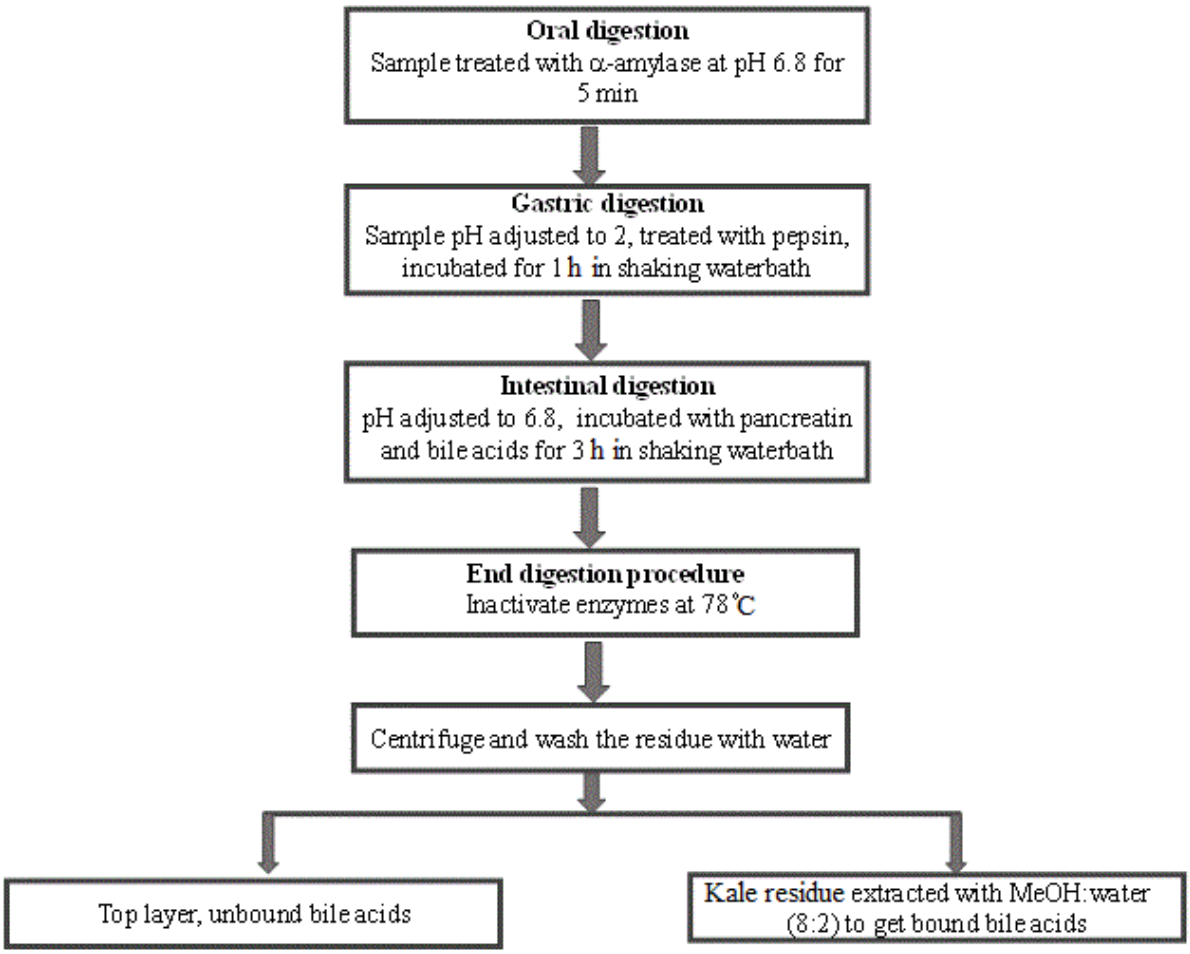

Figure 1. Schematic representation of measurement of *in vitro* bile acid binding capacity of different vegetables, and extraction of bound bile acids

In vitro *Digestion of Different Bile Acid Compositions*

Bile acid composition differs among individuals depending on their health condition and gender. To examine whether the binding capacity is influenced by the composition of the bile acid mixture, various bile acid compositions were used in the experiment to mimic that of male patients with gallstones (BAC-1), healthy females (BAC-2), and males with type 2 diabetes (BAC-3).[57, 58] Mixtures with three different bile acid compositions (BAC) were prepared as shown in Table S2. The BAC-1 bile acid mixture contained 33.3% cholic acid together with glycocholic acid, 24.9% deoxycholic acid together with glycodeoxycholic acid, and 41.8% chenodeoxycholic acid together with chenodeoxycholic acid. The BAC-2 mixture contained 33.1% cholic acid in both conjugated and unconjugated form, 17% deoxycholic acid and its glycine conjugated form, and 49.9% chenodeoxycholic acid together with glycochenodeoxycholic acid. The BAC-3 mixture contained 33.3% cholic acid (conjugated and unconjugated), 39.3% deoxycholic acid (conjugated and unconjugated), and 27.4% chenodeoxycholic acid (conjugated and unconjugated). Lyophilized green kale was used in the previously described *in vitro* digestion protocol and during the intestinal digestion phase, 4 ml of the three bile acid mixtures with different compositions were used. Except the bile acid mixtures, other parts of the *in vitro* experiment were the same as shown in Figure 1. After *in vitro* digestion, the supernatant was concentrated by rotary evaporation and filtered through a cellulose filter for HPLC analysis.

Impact of Different Kale Parts on in vitro Bile Acid Binding Capacity

Lyophilized kale stem, kale leaf, and whole kale were tested for *in vitro* bile acid binding capacity, according to the above protocol except during the intestinal digestion phase, the

bile acid mixture contained taurocholate (4.84 mM), cholate (13.9 mM) and deoxycholate (6.16 mM). After the *in vitro* digestion, the supernatant was concentrated to achieve the minimal volume under vacuum, and passed through a cellulose filter prior to HPLC analysis.

Optimization of Different Doses of Kale for Bile Acid Binding

Kale was found to have the highest bile acid binding capacity among the tested vegetables. To determine the minimal kale dose needed to bind the maximum amount of bile acids, different weights of kale (0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.5 g) were in another *in vitro* experiment. For this experiment, in the intestinal digestion phase, the bile acid mixture BAC-3 was used to mimic the bile acid profile of a diabetic male. All other procedures were the same as those described before.

Minimal Processing of Green Kale for Bile Acid Binding

To understand the impact of mild processing on kale bile acid binding ability, two minimal processing techniques were used:

a). Microwave processing. Fresh-cut kale (18.3 g equivalent to 2.0 g of dried sample) was weighed into three 150 ml beakers and 20 ml of water was added to the vegetables and microwaved for 1, 2, and 3 min respectively prior to the experiment. All samples were processed in triplicate, then homogenized to a paste for 2–3 min.

b). Steaming. Fresh-cut kale (18.3 g) was placed in a 150-ml beaker and steaming was performed for 8 and 10 min at 95°C using a water bath. Analysis of both the time points were conducted in triplicate and used to test the bile acid binding capacity of the homogenized cooked kale.

Extraction of Bound Bile Acids

Digested residue kale (2.0 g) from section 2.6, was extracted with methanol:water:formic acid (80:19:1), followed by 2 min homogenization, 2 h sonication at 60°C, centrifuged for 30 min and filtered through Whatman filter paper. The residue was re-extracted with the same solvent, using 10 ml of solvent as mentioned above, and combined and concentrated by rotary evaporation at 40°C under reduced pressure to obtain 5–10 ml and analyzed by HPLC.

Quantification of Bile Acids by HPLC

The HPLC system consisted of an Agilent HPLC 1200 series (Foster City, CA) with a degasser, quaternary pump, auto-sampler, column oven, and photodiode array detector. Elution of bile acids was carried out with gradient mobile phases of (A) 0.3 M phosphoric acid in water and (B) acetonitrile with a flow rate of 0.8 ml/min at 30°C using Gemini C18 (Phenomenex, Torrance, CA) column. Bile acids were separated using the gradient, 75% to 45% A from 0 to 10 min, 45% to 10% in 10–20 min, 10% to 75% in 20–25 min, maintained isocratic flow for 5 min. Data were processed using the CHEM-STATION software (Agilent, Foster City, CA). All six bile acids at different concentrations were injected to obtain the area. The calibration graphs were prepared by area versus different concentration (31.25 to 1000 ppm) of bile acids and regression equations are presented in Table 1. Bound bile acids samples were injected and the presence of bile acids in each sample was examined by comparing the retention times with those of the standard bile acids. This assay was conducted in triplicate with three independent experiments and results were averaged.

Table 1. Calibration curves used for quantification of bound bile acids

Bile acid	Equation	Dose (mg)	R_2
Glycocholate	y = 83017x + 52.71	2.42	0.99
Cholate	y = 9990.8x + 3.15	22.72	0.99
Glycochenodeoxycholate	y = 77178x + 31.06	9.68	0.99
Glycodeoxycholate	y = 73395x + 25.22	4.84	0.99
Chenodeoxycholate	y = 12607x - 22.76	9.68	0.99
Deoxycholate	y = 11559x + 3.77	22.72	0.99
Taurocholate	y=21149x+23.42	12.76	0.99

Identification of Bile Acids by LC-MS

Bile acids were identified by ultra-high-performance liquid chromatography time of flight-mass spectrometry (LC QTOF–MS) (Maxis Impact, Bruker Daltonics, Billerica, MA). Bound bile acid samples were separated on a Zorbax Eclipse Plus C18 rapid resolution column (1.8 microns partial size, 100 × 2.1 mm) using an Agilent 1290 UHPLC instrument (Agilent, Waldbronn, Germany). The separation was carried out at 65°C with a flow rate of 0.2 ml/min using gradient elution with increasing strength of acetonitrile in 0.1% formic acid. Mass spectral analyses were performed using ESI-Q-TOF mass spectrometer equipped with an electrospray ionization source in positive ion mode. Capillary voltage was maintained at 2.9 kV, source temperature was set at 65°C and nitrogen was used as the desolvation gas (12 l/min).

Fiber Analysis

Lyophilized green kale, red kale, red cabbage, red leaf lettuce, and Brussels sprouts were analyzed for total dietary fiber, soluble fiber, and insoluble fiber according to AOAC protocols by Medallion labs, Minneapolis.

Statistical Analysis

All results were expressed as means ± SE. The data were evaluated by JMP Pro 12 and probability of $p < 0.05$ was considered as statistically significant. Triplicate analyses were conducted for each sample.

Results and Discussion

Bile Acid Binding Capacities of Leafy Vegetables

Binding bile acids has multiple health benefits.[5, 54, 56] Although some vegetables have been tested for *in vitro* bile acid binding capacity, we lack comprehensive data on bile acid binding with reference to dose and levels of individual bile acids typical of different health conditions. The present study compared the bile acid binding capacities of different vegetables and used the vegetable with the best bile acid binding to test its effectiveness using various compositions of bile acids and the different doses. Figure 2 shows that the green and red kale exhibited the highest bile acid binding capacity, 87% and 90%, respectively. Among the five tested bile acids, only cholic acid showed the lowest binding capacity by kale. The rest of the vegetables, such as red leaf lettuce (78%) exhibited significantly higher binding than Brussels sprout (62%) and red cabbage (58%). Deoxycholic acid and chenodeoxycholic acid are the more hydrophobic bile acids, and may induce colon

cancer and liver stress,[59, 60] but cholic acid is more hydrophilic and its liver toxicity is milder than that of the hydrophobic bile acids. The bile acid binding capacities of five vegetables were, from the highest to the lowest: red kale, green kale, red leaf lettuce, Brussels sprouts, and red cabbage. Table S3 shows the moisture content and fresh weight equivalent of the vegetables used for the bile acid binding assay. The difference in bile acid binding between various green vegetables may be due to their different chemical compositions, including phenolics, glucosinolates, flavonoids, dietary fiber, and other primary metabolites. The moisture content in Brussels sprouts and red leaf lettuce was the lowest (84.4%) and highest (95.6%), respectively. To get the bile acid binding capacity shown in this *in vitro* experiment, it required 15.7 g red kale, 15.4 g green kale, 45.9 g red leaf lettuce, 12.8 g Brussels sprouts, and 23.0 g red cabbage. Kale bound more chenodexoycholic acid and deoxycholic acid than red cabbage, Brussels sprouts and red leaf lettuce. Kale also had the highest total bile acid binding capacity relative to Brussels sprouts, red leaf lettuce. Although, the bile acid binding capacity of green kale and red kale showed no significant difference from each other, the literature shows that green kale has 81% more flavonols and 140% more glucosinolates than red kale.[61] Therefore, we used green kale to determine the exact dose needed for maximum bile acid binding capacity.

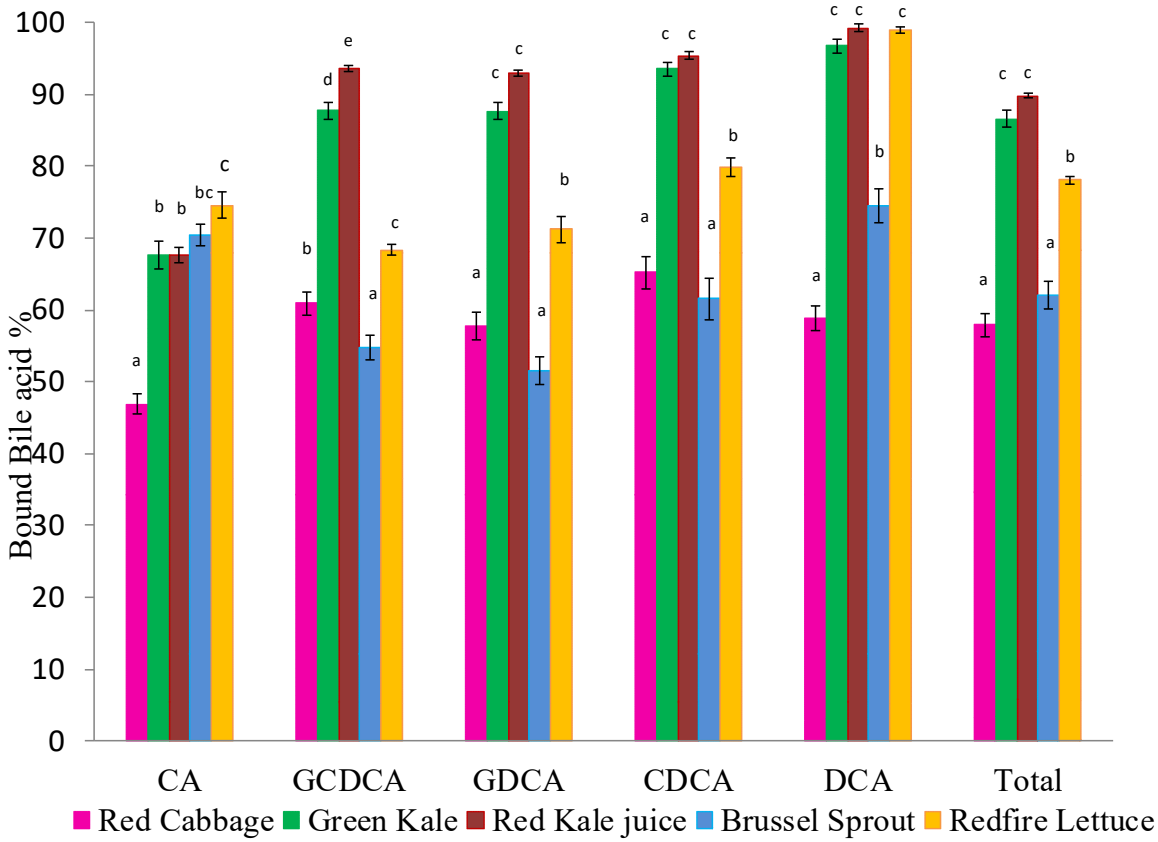

Figure 2. Bile acid binding capacity of total bile acids and individual bile acids for different vegetables. Tested were: red cabbage, green kale, red kale, Brussels sprouts, red leaf lettuce and cholate (CA), glycochenodeoxycholate (GCDCA), glycodeoxycholate (GDCA), chenodeoxycholate (CDCA), deoxycholate (DCA). Each value is the mean taken from 3 replicates, with error bars for the standard error. Different letters (a, b, c) indicate that the difference between two values is significant on the level $p<0.05$.

In Vitro *Digestion of Green Kale with Different Bile Acid Compositions*

The composition of the bile acids varies among individuals, based on different health conditions, and is intrinsically linked to their physiology. This occurs via a variety of regulatory processes, difference in the ratios of primary versus secondary bile acids, and the microbial ecology of the gut.[62-64] To understand bile acid compositions impact on the *in vitro* bile acid binding capacity of kale, we used three bile acids compositions to simulate different health conditions, including male with gallstones (BAC-1), healthy female (BAC-2) and

male with type-2 diabetes (BAC-3) (Table S2). The results showed that BAC-1 (male with gallstone) and BAC-2 (healthy female) were bound in significantly higher amounts by kale than BAC-3 (type-2 diabetic male model) (Figure 3). The binding capacity of deoxycholic acid remained the same in all three compositions. In the type-2 diabetic male model, green kale bound significantly less GDCA, GCDCA, GC, and left more conjugated bile acids to protect the liver. According to Ho et al.[65] the human bile pool size is 3055 mg, and Brufau et al.[18, 57] reported no significant difference of total bile pool size between healthy individuals and type-2 diabetic patients. Thus, we considered 3055 mg as the total bile acids for the calculation of both the healthy model and the type 2 diabetes model.

The U.S. Food and Drug Administration has approved a bile acid sequestrant, colesevelam hydrochloride, for the treatment of type-2 diabetes. Clinical trials demonstrated that colesvelam significantly improves the effect of three different drugs (metformin, sulfonylurea, and insulin) for type-2 diabetes. The addition of colesvelam involved in these treatments helps to reduce HbA1C from -0.32% to -0.41%.[66] The mechanism by which colesvelam lowers blood glucose level is not yet established, however, it might be related to the farnesoid X receptor (FXR) and the G protein-coupled receptor TGR5. The bile acid receptor FXR is involved in both lipid and glucose metabolism, and FXR can inhibit hepatic glucose production. Meanwhile, bile acids can induce G protein-coupled receptor TGR5, which can increase energy expenditure.[67, 68] Therefore, green kale may provide health benefit to blood sugar control for type 2 diabetic patients.

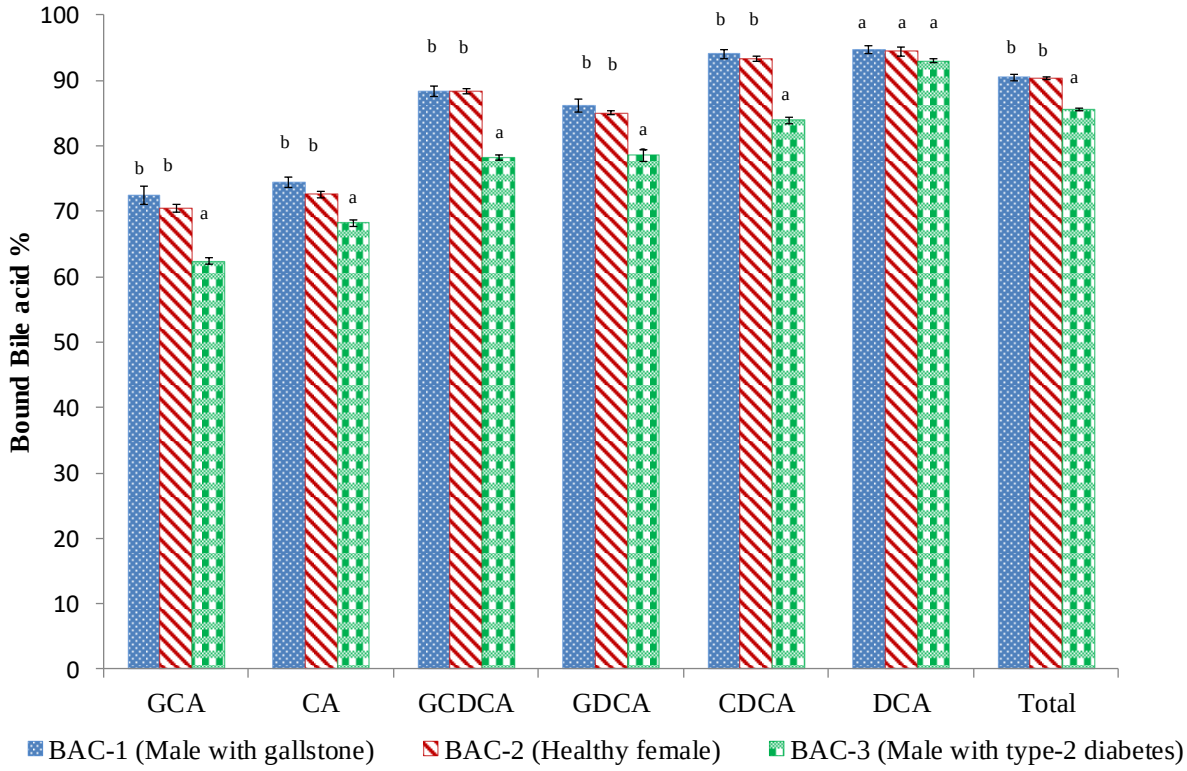

Figure 3. Green kale bile acid binding capacity in three different models of bile acid composition: male with gallstone (BAC-1), healthy female (BAC-2), type 2 male diabetic patients (BAC-3). Each value is the mean taken from 3 replicates, with error bars for the standard error. Different letters (a, b) indicates that the difference between two values is significant on the level $p<0.05$. Each bile acid binding capacity were compared between different bile acid compositions.

In Vitro *Bile Acid Binding of Different Parts of Kale*

Most of the time, kale leaf or whole kale is consumed. To understand the potential health benefits of kale, lyophilized kale leaf, kale stem, and whole kale were tested for *in vitro* bile acid binding. As seen in **Figure 4**, kale stem binds less taurocholate than kale leaf and whole kale, while it bound similar cholate, deoxycholate and total bile acid compared to kale leaf and whole kale. Additionally, whole kale bound more bile acids than kale stem and kale leaf. The kale stem bound 63.0% of total bile acids, including 86.3% of deoxycholate, 56.7% of

cholate and 54.8% of taurocholate. The kale leaf bound 65.2% of total bile acids with 91.4% of deoxycholate, 56.9% of cholate, and 58.4% of taurocholate. The whole kale sample bound 67.4% of total bile acids, 93.8% of deoxycholate, 59.4% of cholate and 59.4% of taurocholate. Therefore, it is not necessary to consume kale stems for bile acid binding, and whole kale has the best bile acid binding capacity.

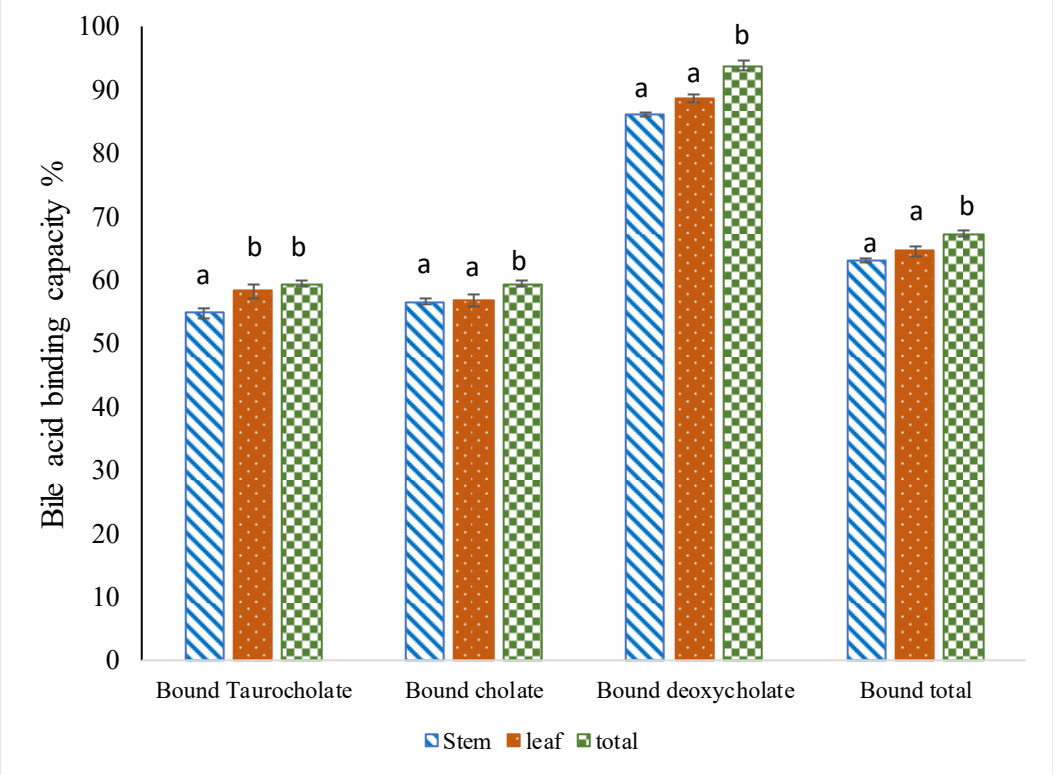

Figure 4. Bile acid binding capacity of different parts of kale. Each value is the mean taken from 3 replicates, with error bars for the standard error. Different letters (a, b) indicates that the difference between two values is significant on the level $p<0.05$. Each bile acid binding capacity were compared between kale stem, kale leaf and whole kale.

Optimization of Different Doses of Kale for Bile Acid Binding Capacity

To estimate the optimal dose for green kale to achieve the maximum bile acid binding capacity, different doses of green kale were tested *in vitro*. The portion of bound bile acids

has correlation increased with the dose of the freeze-dried green kale powder before reaching the saturate amount. As shown in Figure 5, the samples had limited binding capacities for each individual bile acid and for total bile acids. Also, each bile acid has its own optimal amount for the highest binding capacity; beyond this dose, even if more green kale was added into the *in vitro* digestion, the binding ratio of bile acids did not increase. Table 2 shows the logistic regression curve explaining the relationship between kale dose and bile acid binding capacity, general form as $Y=0.95A_1$. The limits for each individual and total bile acid binding capacity ranged from 66–94%. It is commonly stated that the threshold is the value of X at which the corresponding Y value is 5% below the upper plateau. Hence the threshold point of each logistic equation is calculated as $X=A_3-2.944/A_2$, while $Y=0.95A_1$, according to the equation derived by McDowall. [69] The threshold of each individual bile acid displayed in table 2 suggested that the optimal dose of kale required was 1.56 g for GCA, 1.75 g for CA, 1.49 g for GCDCA, 1.29 g for GDCA, 1.24 g for CDCA, 0.69 g for DCA, and 1.31 g for total bile acids (Table 2). To compare results of kale bile acid binding capacity, cholestyramine resin was tested and it bound 93.45% of total bile acids at 0.33 g (Figure 5).

In clinical trials of different individuals, Moutafis et al[41] measured the fecal excretion of bile acids (1897.76 mg) in response to a dose of 30 g of cholestyramine. The logistic equation of this present experiment for cholestyramine is Y=98.36458/(1+exp(-11.570799*(X+0.077039))), where Y refers to the percentage of bound bile acids and X refers to the dose (g) of cholestyramine. The total input of bile acids in this *in vitro* experiment is 72.06 mg, while the bile pool of the human body is 3055 mg, which is 42.4 times the total used in this experiment. Supposing the *in vitro* bile acid binding capacity

can be extrapolated to the human body, 30 g of cholestyramine taken by the individual in the clinical trial is equivalent to 0.7075 g cholestyramine in this *in vitro* experiment, and according to the logistic regression equation obtained in this experiment, about 98.35% total bile acids can be bound. If this *in vitro* experiment can be extrapolated to the human body, about 3004.53 mg bile acids should be excreted into the human feces. However, Brufau et al. (2010)[26] reported that 1897.76 mg bile acids were excreted with 30 g of cholestyramine daily intake, hence there is a factor linking this *in vitro* experiment and the clinical trial, which is 0.6316, (1897.76/3004.53=0.6316). This *in vitro* experiment determined that no more than 81.81% of total bile acids in human[36] can be bound, which, adjusted by the factor 0.6316 yields the result that 1579.98 mg bile acids can be excreted by consuming green kale. Extrapolated to the human body, to achieve 99% of the limit of bile acid excretion, a person must consume 55.6 g dry green kale, or approximately 427.0 g fresh green kale. A previous study reported that additional bile acid sequestrant significantly improved the reduction of HbA1c and LDL cholesterol by insulin-based therapy.[42] Therefore, for type-2 diabetes patients, consuming more green kale daily may help to improve insulin-based treatment for control of their blood sugar. Since 427.0 g fresh green kale is not a practical dose for daily consumption, the widely available dried kale snacks may help increase kale intake.

The previously determined doses in Table 2 can be used as a reference for future *in vivo* experiments for bile acid binding and the result could vary from this *in vitro* experiment due to additional factors that involved in the digestive system. Intestinal bacteria are involved in the biotransformation of bile salts and may alter the bile acid composition. Certain bacteria could also increase the hydrophobicity of bile salts.[16] For instance, *Lactobacillus acidophilus* in the human intestinal tract can deconjugate glycocholate and taurocholate, which increases

the CA content in the digestive tract.[70] Moreover, the bile acids bound by vegetables in this *in vitro* digestion may not be permanently bound in human digestive system. In Dziedzic's research, it is indicated that during large intestine digestion phase, free bile acids — cholic acid, deoxycholic acid and lithocholic acid — increased, compared to the small intestinal digestion phase. [71] Several factors may contribute to the increased bile acids in large intestine. One of the reason probably is due to the bacteria hydrolysis effect on dietary fibers and liberate the bound bile acids. Another explanation is the common faecal bacteria, *E. coli*, has cytochrome P450 enzyme, which is similar to the enzymes from hepatocytes. Therefore, *E. coli* may be able biotransform cholesterol or phytosterols to primary bile acids. [72, 73] In addition, human intestinal bacteria can also transform primary bile acids into secondary bile acids. *Clostridium scindens, Clostridium hiranonis, Clostridium hylemonae*, and *Clostridium sordelli* can convert DCA from CA and LCA from CDCA respectively.[15, 16]

Secondary bile acids cannot be absorbed by the liver through the portal vein and therefore they accumulate in the bile pool.[16] Deoxycholic acid content in the bile acid pool can be up to 75% in some individuals favoring a Western diet.[15-17] Moreover, deoxycholic acid can induce oxidative stress in colon cells, increase the risk of gene mutation, and eventually may promote the development of tumors or cancer.[4] Berstein et al. (2011) reported that deoxycholic acid induced 94% of colon tumors in mice used in the experiment, including 56% of colon cancers.[39] In the present study, 104.2 g of green kale was able to bind 93.71% of the deoxycholic acid, indicating that kale consumption may have the potential to reduce colon cancer risk, which should be explored in future studies.

Table 2. Logistic regression curve for individual bile acid binding capacity in response to kale dose

X: refers to green kale dose (g)

Y: refers to bile acid binding percentage (%)

Bile salt	Equation	Saturation points	
		X value	Y value
Glycocholate	Y=67.472958/(1+Exp(-2.2221099*(X-0.2364677)))	1.561	64.1
Cholate	Y=70.21116/(1+Exp(-2.7243291*(X-0.6714658)))	1.752	66.7
Glycochenodeoxycholate	Y=81.507683/(1+Exp(-3.0382878*(X-0.5179148)))	1.487	77.4
Glycodeoxycholate	Y=81.055733 /(1+Exp(-4.011064*(X-0.5510876)))	1.285	77.0
Chenodeoxycholate	Y=87.215157/(1+Exp(-3.6301377*(X-0.4235821)))	1.235	82.9
Deoxycholate	Y=94.784765/(1+Exp(-7.5850513*(X-0.3074524)))	0.696	90.0
Total Bile Acids	Y=81.883651/(1+Exp(-3.1635124*(X-0.3817649)))	1.312	77.8

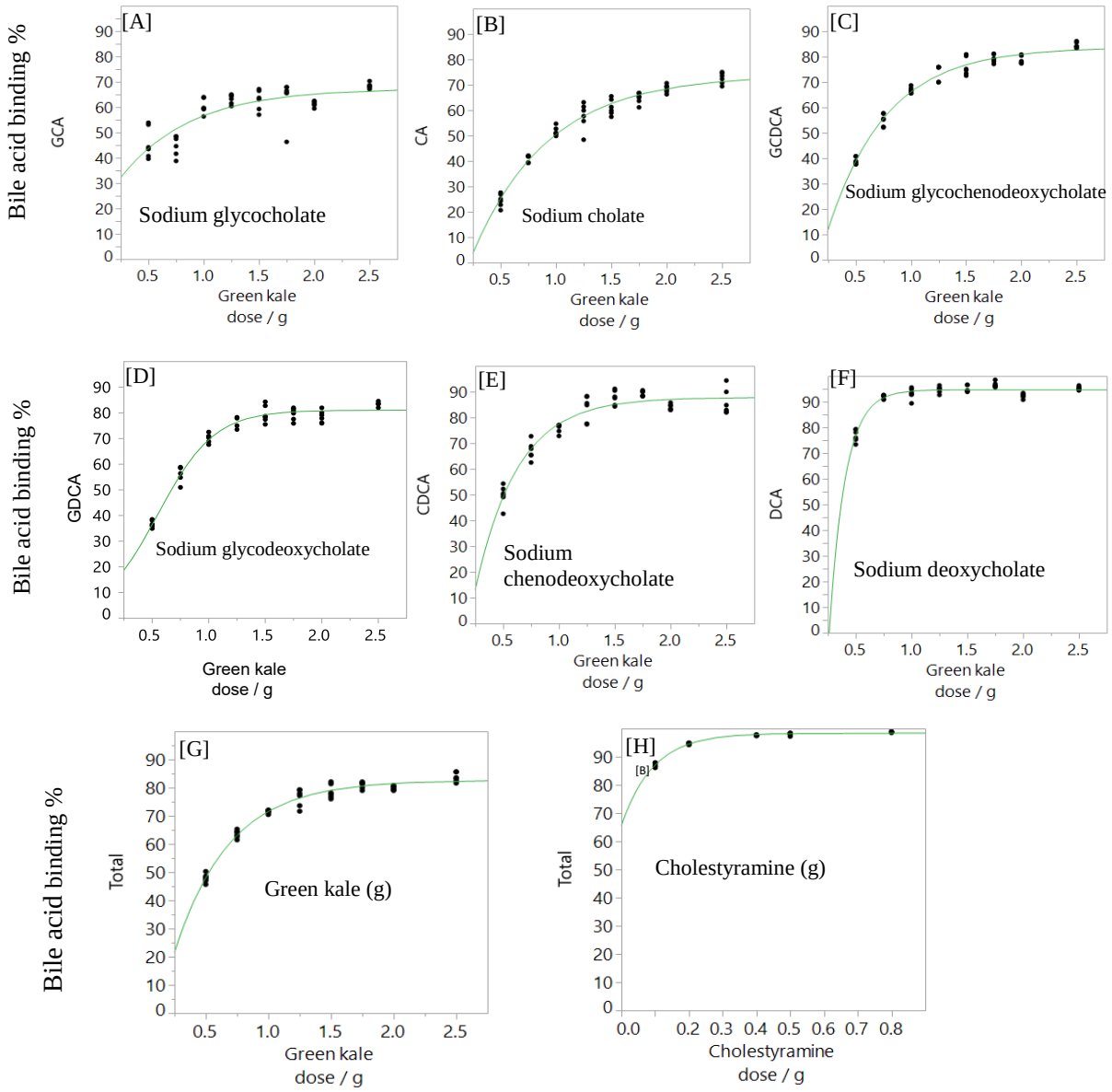

Figure 5. Bile acid binding capacity in response to input dose of kale leaf: (A) Glycocholate; (B) Cholate; (C) Glycochenodeoxycholate; (D) Glycodeoxycholate; (E) Chenodeoxycholate; (F) Deoxycholate; (G) Total bile acids; (H) Total bile acids binding capacity in response to cholestyramine dose. Bile acid binding capacity was measured as the percentage of bound bile acids.

Minimally Processed Green Kale Bile Acid Binding Capacity

Fresh kale (18.3 g, equivalent to 2.0 g lyophilized kale) was tested for the effects of minimal processing by microwaving or steaming. As shown in Figure 6, minimal processing significantly improved kale's *in vitro* binding capacity for glycocholate, cholate, glycochenodeoxycholate, and glycodeoxycholate. Also, minimally cooking slightly improved kale's binding capacity for chenodeoxycholate, deoxycholate, and total bile acids. However, the cooking methods showed no significant difference in bile acid binding. Cooked kale bound 90% of total bile acids. There is no significant difference in bile acid binding capacity between microwaved kale and steamed kale. However, 2 min's microwave cooking bound slightly less glycochenodeoxycholic acid and deoxycholic acid than other cooking methods. Microwaved kale (2 min) bound 93% deoxycholic acid, while microwaving 1 min and 3 min to kale bound 97% deoxycholic acid. Steamed kale bound similar amount of total bile acids as microwave cooked kale. Yet, steamed kale bound significantly less chenodeoxycholic acid than 1 min's and 3 min's microwaved kale. Therefore, microwave cooking for 1 min or 3 min can offer kale with best *in vitro* bile acids binding capacity. This could result from the changes in the structure of protein and fiber, which increases the number of active binding sites and thus enhances bile acid binding capacity. In addition, cooking may release some lipids, which promotes bile acid binding capacity. Kalhon et al[5] observed similar results, but provided no report of binding of individual bile acids.

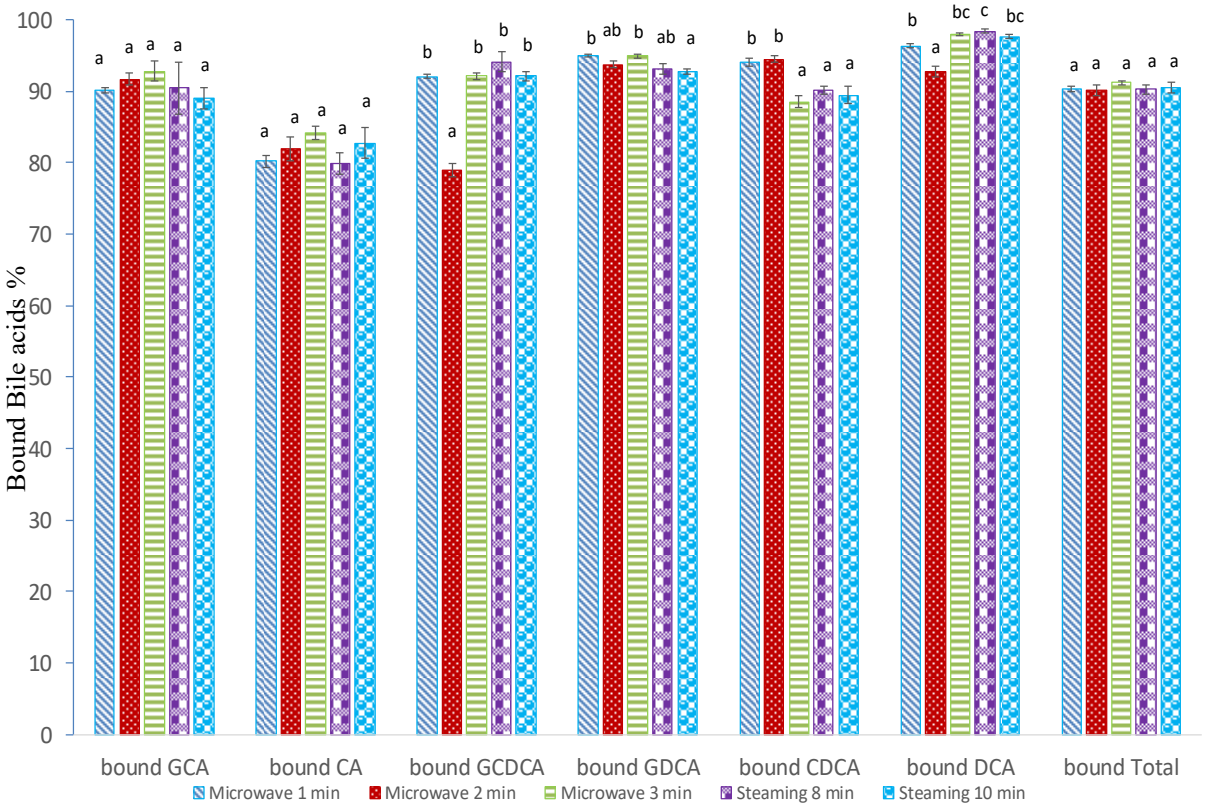

Figure 6. Bile acids binding capacity of kale treated with different cooking methods, compared with lyophilized kale. Each value is the mean taken from 3 replicates, with error bars for the standard error. Different letters (a, b, c) indicate that the difference between two values is significant on the level $p<0.05$.

Relationship Between Fiber Content and Bile Acid Binding Capacity

Total dietary fiber, soluble fiber, and insoluble fiber contents were measured (Table S3). The total fiber ranged between 40% and 30.3%. Each gram of insoluble fiber in these vegetables bound 93.1–138.7 mg of total bile acids. On comparing the bile acid binding capacity with fiber types, we found that soluble fiber binds more bile acids than insoluble fiber. Dietary fibers (pectin and gum) can swell and their surface area will increase in an aqueous system, which may enhance the bile acid binding capacity.[74] Each gram of dietary fiber from red leaf lettuce binds 109.2 mg of bile acid. Therefore, among these five vegetables, the total bile acid binding capacity cannot be related to the total dietary fiber content. Kahlon et al[5] reported the bile acid binding capacity using various vegetables and

the activity has been attributed to bioactive compounds present in each crop, along with total dietary fiber content and type of fibers. In the present study, total bile acid binding capacity based on the insoluble fiber (ISF) were red leaf lettuce, followed by red kale, green kale, Brussels sprouts, and red cabbage. Each gram of insoluble fiber in these vegetables bound 93.1 to 138.7 mg of total bile acids.

Comparison of the bile acid binding capacity based on soluble and insoluble fiber showed that soluble fiber binds much more bile acids than insoluble fiber. Soluble fiber mainly includes pectin, gum, and hemicellulose; insoluble fiber mainly includes hemicellulose and cellulose. Gum and pectin have good *in vitro* bile acid binding capacity, but cellulose does not bind bile acids very well.[5, 47, 75] The result of individual bile acid binding capacity tests of dietary fiber from five vegetables indicated that glycochenodeoxycholic acid was bound mostly by dietary fiber.

Confirmation of Bile Acids

To confirm the bound bile acids, the digested residue of each vegetable was extracted with MeOH:water:formic acid (80:19:1) and analyzed by high resolution mass spectrometry. Figure 7A shows the total ion chromatogram and broad band collision-induced dissociation (bbCID) mass spectra of five bile acids separated in 8 min. Figure 7 (B–F) shows the accurate mass spectra of molecular ions obtained by electrospray negative ionization mode for each bile acids along with their bbCID spectra. All the molecular weights matched those of their respective molecular ions, which confirms the presence of intact bile acids without degradation.

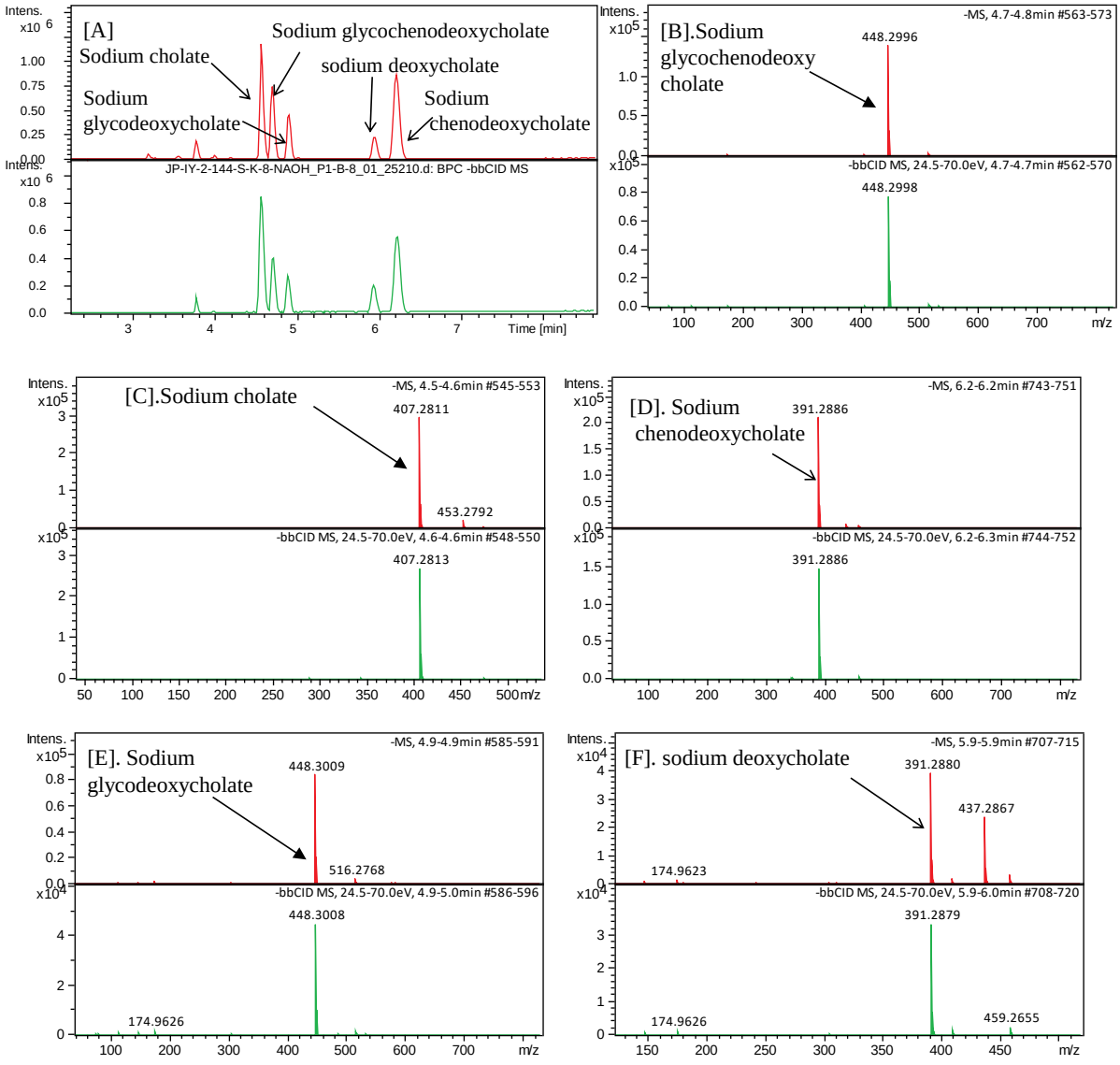

Figure 7. Confirmation of bile acids by LC-MS, [A]. Total ion and broadband collision-induced dissociation (bbCID) chromatogram of bound bile acids separated on eclipse plus C_{18} column.
[B-F]. Mass spectra of bound bile acids confirmed by high resolution time of flight by electrospray negative ionization mode.

Conclusion

Red kale and green kale have higher *in vitro* bile acid binding capacity than red cabbage, red leaf lettuce, and Brussels sprouts. Green kale was equally effective at binding bile acids, when the BA composition simulated a healthy female or a male with gallstone, and it bound slightly lower amounts of bile acids, when the profile simulated bile acids in a type 2 diabetic male. In the bile acid mixture mimicking type-2 diabetic patients, green kale bound no more than 81.8% of the bile acids, which was accomplished by a dose of 1.31 g (dried weight). Converted to the human body, as much as 51.7% of bile acids can be excreted by consuming 55.6 g green kale (dry matter), which is about 427.0 g of fresh green kale. Green kale preferentially bound the hydrophobic bile acids deoxycholic acid and chenodeoxycholic acid. The recommended green kale dose demonstrated the ideal bile acid binding in the *in vitro* experiment; this dose can be used as a reference for future *in vivo* experiments or even clinical trials. In addition, steaming and microwave heating improved kale's bile acid binding capacity.

CHAPTER IV

ENHANCED BIOACCESSIBILITY OF KALE POLYPHENOLS BY BILE ACIDS INVOLVED IN THE *IN VITRO* DIGESTION

Kale has abundant dietary fiber and polyphenols, mainly derivatives of kaempferol and quercetin. Quercetin and kaempferol have antiproliferative activities on colon cancer cells. Dietary fiber can bind bile acids, but whether the polyphenols in kale contribute to its *in vitro* bile acid binding capacity remains unknown. Also, there is no established theory about how bile acids interact with kale's bioactive compounds, including dietary fiber and polyphenols. In this study, polyphenols were separated from the fiber-rich tissue in kale (*Brassica oleracea*) through Soxhlet extraction, and then were incubated with bile acids. Raw kale was incubated with bile acid mixtures during *in vitro* digestion, and the bile acid binding capacity was compared with that of the fiber-rich tissue in the Soxhlet-extracted residue. Finally, lyophilized raw kale was digested *in vitro* with and without bile acids. These results indicated that the fiber-rich tissue in kale binds the most bile acids. Polyphenols in kale do not attach to bile acids after the incubation, but they may interact with bile acids through intermolecular interactions. Bile acids involved in the *in vitro* digestion can change the polyphenol profile of kale. Raw kale (1.0 g) contains 2.19 mg of quercetin derivatives, 0.98 mg of kaempferol derivatives, and a total of 3.15 mg of identified polyphenols. When bile acids were added to the *in vitro* digestion, 63.8% of total identified polyphenols were bioaccessible, 60.1% of quercetin derivatives were bioaccessible, and 70.2% of kaempferol derivatives were bioaccessible. Without bile acids in the *in vitro* digestion, the bioaccessibility of total identified polyphenols and kaempferol derivatives was 14.5% and 3.3%, respectively, but

only 39.1% of quercetin derivatives were bioaccessible. Therefore, bile acids can significantly improve the bioaccessibility of quercetin derivatives and total identified polyphenols, but slightly decrease the bioaccessibility of kaempferol derivatives.

Introduction

Kale (*Brassica oleracea*) is a cruciferous vegetable and has won consumers' favor for its antioxidant capacity, anti-cancer potential, and possible anti-aging function. It can be consumed in salads, soups, or roasted as a snack. Kale has a high amount of dietary fiber and various other nutrients, including polyphenols, carotenoids, and minerals. The excellent antioxidant capacity of kale results, in part, from the presence of numerous types of polyphenols, mainly derivatives of kaempferol and quercetin, plus some phenolic acids, such as caffeoylquinic acid, p-coumaroylquinic acid, and feruloylquinic acid.[22] Kaempferol and quercetin in kale have been identified as outstanding antioxidants than can suppress cancer and potentially slow down the aging process.[76] Quercetin can inhibit α-amylase and α-glucosidase, which can help control blood sugar levels for
type-2 diabetic patients.[77] Kale also has some carotenoids, including β-carotene and lutein, which have a protective function for our eyes. [9, 10]

Abundant valuable nutrients exist in kale, yet not all them can be absorbed. For example, some nutrients can be trapped in the plant cells or bound by the indigestible fiber during digestion. Bioaccessibility measures the fraction of a nutrient that is released from the food during digestion. Other nutrients can be released from the food matrix during digestion, enter the gastric intestinal tract,[34] enter the bloodstream, reach a specific location in the human body, and become available at the site of action. Although some compounds may not show

very high bioavailability during in vivo digestion, they may still have health benefits. For instance, the bioavailability of kaempferol is approximately only 2%; [35] however, the consumption of kaempferol improves the cytotoxicity of cisplatin, increasing its anticancer power. [78 79] Hence, it is possible that the anticancer property of kaempferol is more related to its bioaccessibility rather than its bioavailability. Since kaempferol derivatives and quercetin derivatives are the main polyphenols in kale,[8] it is essential to study their bioaccessibilities and their interactions with other chemicals.

Besides polyphenols, kale also contains a large amount of dietary fiber, which can maintain a healthy intestinal microbiota and normal bowel movements. Dietary fiber is also considered to have a protective effect against colon cancer because it can promote the growth of health-beneficial microbes in the intestine and maintain the bulk and moisture of excretion. [80] Besides preventing colon cancer, dietary fiber can bind and sequester bile acids. [81] Kahlon et al (2007) examined the *in vitro* bile acid binding capacity of various vegetables, including kale, broccoli, and Brussels sprouts, and found that kale displayed strong bile acid binding capacity.[5] Interestingly, the same amount of dietary fiber from different vegetables showed different bile acid binding capacities. For instance, 100 mg of total dietary fiber from kale bound 2.27 μmol of bile acids, and 100 mg of total dietary fiber from cabbage only bound 0.81 μmol bile acids. [5] Therefore, other bioactive compounds, such as polyphenols or carotenoids, may influence the bile acid binding capacity of dietary fiber. Bile acids in the digestive system could also interact with polyphenols or carotenoids and affect their bioaccessibilities. However, the interaction between bile acids and various bioactive compounds in kale has not yet been studied. The present study was designed to determine the main bile acid binding components in kale, and examine the potential impact of bile acids on

kale's bioactive compounds. The bioactive compounds in kale, polyphenols and fiber, were separated through Soxhlet extraction, and were incubated with bile acids to determine their interactions. Raw kale also went through the *in vitro* digestion process both with and without bile acids, in order to study bile acids' impact on the bioaccessibility of kale polyphenols.

Materials and Methods

Plant Materials and Reagents

Green kale was purchased from the local market, cut into half inch squares, frozen at -18°C and lyophilized.

Hexane, acetone, and methanol were used for extraction. LC-MS grade methanol, water, and acetonitrile were used for analytical work. All the solvents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Extraction of Phytochemicals from Kale

The lyophilized kale sample (2.0 g) was added to 35 ml extraction solution MeOH : water : formic acid (80:19:1), followed by 2 min of homogenization and 2 h of sonication at 70°C. The extract was centrifuged (BeckMan Model TJ-6), filtered and the residue was re-extracted with 20 ml of the same solvent as previously used. Both extracts were pooled, filtered through Whatman filter paper, and concentrated under vacuum using a rotary evaporator (Buchi) to obtain a sample of around 10 ml, which was then filtered through a cotton cartridge filter for further LC-MS analysis.

Soxhlet Extraction from Raw Kale for Bile Acid Binding Ability

Lyophilized kale (209 g) was loaded into the Soxhlet type apparatus and extracted with hexane (2 l) at boiling temperature for 48 h for the removal of fatty material. The residue was reextracted successively with acetone and MeOH: water: formic acid (80:19:1) separately for 48 h each. All the extracts were filtered and concentrated separately with a vacuum evaporation apparatus to obtain the minimal volume and remove traces of solvents, and lyophilized to obtain dried extract. The yields of hexane, acetone, and MeOH: water: formic acid (80:19:1) extracts were 7.17 g, 6.36 g, and 39.34 g, respectively. The MeOH: water: formic acid fraction was dissolved in water with 1% formic acid and 5% acetone, to 5000 ppm. All the extracted fractions were used for further LC-MS analysis. The leftover kale residue (fiber-rich tissue) was removed from the Soxhlet extraction apparatus, air dried, and used for bile acid binding capacity assays.

The digestion process involved simulated oral digestion, gastric digestion, and small intestinal digestion. For the oral digestion, simulated saliva fluid (SSF) components (see Table S1) were dissolved in phosphate buffer (pH 6.8). Soxhlet-extracted solvent extracts (0.1 g) were mixed with 0.1 ml of DMSO and 1 ml simulated saliva fluid and vortexed, followed by water bath shaking at 37°C for 5 min, at 180 rpm. For gastric digestion, the chyme pH was adjusted to 2.0 with 1 N HCl, then 60 µl pepsin buffer (200 µg pepsin in 1 ml 0.1 M HCl) was added to each sample, followed by vortexing and water bath shaking at 37°C for 1 h. For intestinal digestion, the chyme pH was adjusted to 6.8 with 1 N NaOH, followed by the addition of 0.5 ml pancreatin (6.25 mg/ml in 50 mM phosphate buffer), 0.4 ml bile acid mixture solution (2.54 mM sodium glycodeoxycholate, 12.08 mM sodium cholate, 13.22 mM sodium deoxycholate, 5.14 mM sodium glycochenodeoxycholate, 1.24 mM sodium

glycocholate, 5.84 mM sodium chenodeoxycholate), then vortexing, and water bath shaking at 37°C for 3 h. After the incubation with bile acids, the *in vitro* digestion was terminated by inactivating enzymes at 78°C in a water bath for 7 min. The final digestion fluid was around 2 ml for each digestion sample.

Analytical samples were collected after the termination of *in vitro* digestion. After inactivation of the enzyme, the digestion chyme was centrifuged at 800 g for 30 min, the supernatant was carefully collected and filtered through Whatman 90 mm Ø filter paper. The remaining residue was rinsed with 2 ml nano-pure water in a water bath, with shaking for 2 h.

In vitro *bile acid binding capacity of fiber-rich kale tissue and raw kale*

The fiber-rich kale tissue (0.71 g and 1.0 g) and raw kale (1.0 g) were used for *in vitro* digestion with bile acids. The raw kale and fiber-rich Soxhlet extracted residue samples (in triplicate) were suspended in water in a ratio of 1:1 and 10 ml simulated saliva fluid (components see Table S1), before 0.31 mg α-amylase was added to the samples and then mixed by vortexing for 30 s. The samples were incubated in a shaking water bath at 37°C for 6 min, with a shaking speed of 180 rpm. Then, to stimulate gastric digestion, the chime pH was adjusted to 2.0 with 1 N HCl, and 600 µl pepsin (200 µg pepsin in 1 ml 0.1 M HCl) was added and incubated in a shaking water bath at 37°C for 1 h. Further intestinal digestion was conducted at pH 6.8, followed by the addition of 5 ml pancreatin (6.25 mg/ml in 50 mM phosphate buffer), 4 ml bile acid mixture (2.54 mM sodium glycol deoxycholate, 12.08 mM sodium cholate, 13.22 mM sodium deoxycholate, 5.14 mM sodium glycochenodeoxycholate, 1.24 mM sodium glycocholate, 5.84 mM sodium chenodeoxycholate), then vortexed, and

incubated in a water bath, with shaking, at 37°C for 3 hrs. After the incubation with bile acids, the *in vitro* digestion was terminated by inactivating enzymes at 78°C in a water bath for 7 min. The final digestion reaction was around 20 ml for each sample.

Analytical samples were collected after the termination of *in vitro* digestion. After inactivation of the enzyme, the digestion chyme was centrifuged at 800 g for 30 min, the supernatant was carefully collected and filtered through Whatman filter paper. The remaining residue was rinsed with 30 ml nano-pure water in water bath shaking for 2 h. The two supernatants were combined and rotary evaporated at 40°C to reduce the volume to approximately 10 ml, which was filtered through a cotton cartridge filter and used for HPLC analysis.

Bioaccessible compounds of kale after* in vitro *digestion (with and without bile acids)

Lyophilized kale (1.0 g) was used to conduct another *in vitro* digestion process that included oral, gastric, and intestinal phases. In the oral digestion phase, 0.31 mg α-amylase was added into 10 ml simulated saliva fluid (SSF) with ingredients shown in Table S1, and rest of the digestion process followed as mentioned above. [53-56]

For analysis of bioaccessible compounds, one set of samples was digested with 4 ml buffer replacing the 4 ml bile acid mixture. All samples were centrifuged, washed, pooled, and filtered through a cotton cartridge filter for further LC-MS analysis. The residue was extracted twice with 20 ml of MeOH: water: formic acid (80:19:1), with a 2 min homogenization followed by 2 h sonication, then centrifuged at 800 g for 30 min. Both the supernatants were combined and concentrated under vacuum by rotary evaporation at 40°C and adjusted to a known volume, then filtered through a cotton cartridge for LC-MS

Quantification of Bile Acids by HPLC

Bile acids in the samples were quantified with an Agilent 1200 Series HPLC consisting of a degasser, quaternary pump, auto-sampler and PDA detector at 210 nm. Samples (25 µl) were injected and separation was carried by reversed phase Gemini C18 column with solvent A (30 mM phosphoric acid) and solvent B (acetonitrile). Compounds were eluted as follows, 75% to 45% A from 0 to 10 min, 45% to 10% from 10 to 20 min, 10% to 75% A from 20 to 25 min, isocratic at 75% A from 25 to 30 min. Each individual bile acid was injected at different concentrations (1000 to 12.5 µg/ml) to obtain calibration graphs. Using the individual regression equations, the bound bile acids were quantified using peaks area and dilution factors. This assay was conducted in triplicate with three independent experiments and results were averaged.

Fiber Analysis

Lyophilized raw kale and fiber-rich fractions were analyzed for total dietary fiber, soluble fiber, and insoluble fiber according to the Association of Analytical Communities protocols by Medallion Labs (Minneapolis, MN).

LC-MS Analysis

The digested supernatants, methanol extracts from digested residues, raw kale, and Soxhlet extracts were analyzed by LC-MS. The chemical constituents from all the samples were characterized by ultra-high performance liquid chromatography-time of flight-mass spectrometry (UHLC-QTOF-MS) (maXis impact, Bruker Daltonics, Billerica, MA). The

separation was performed on Zorbax rapid resolution high definition Eclipse Plus C_{18} column (2.1 mm × 100 mm, 1.8 μm particle size). Column temperature was kept at 75℃ with a flow rate of 0.2 ml/min. The mobile phases used for separation were (A) 0.1% formic acid in nano pure water and (B) 0.1% formic acid in acetonitrile (7:3). The column was eluted as follows, 100% A for 0 to 5 min, 100–98% A for 5 to 10 min, 98–55% A from 10 to 22 min, 55–40% A from 22 to 27 min, 40–10% A from 27 to 31 min, isocratic for 31 to 34 min, 10–100% A from 34 to 40 min. The column was equilibrated for 2 min before the next injection. Mass spectral analyses were performed using the quadruple time of flight (Q-TOF) mass spectrometer equipped with an electrospray ionization source in positive ionization mode. MS experiments were carried out under the following conditions, ionization source in positive ionization mode ESI (+); MS scan range 50–1000 m/z; end plate offset 500 V; capillary 3000 V, nebulizer gas (N_2) 0.2 bar; dry gas (N_2) 48 l/min; dry temperature: 200°C; ion transfer conditions funnel RF: 200 Vpp; multiple RF: 200 Vpp; quadruple low mass 55 m/z; collision energy 5.0 eV; collision RF 600 Vpp; ion cooler RF 50–250 Vpp ramping; transfer time 121 μs; and pre-pulse storage time 1 ms. External mass spectrometer calibration was performed using a Cole Palmer syringe pump (Vernon Hills, Illinois, USA) directly connected to the interface, equipped with a Hamilton syringe (Reno, Nevada, USA) containing sodium formate (5 mM sodium hydroxide and water: 2-propanol 1:1 (v/v) with 0.2% of formic acid). The calibration solution was injected at the end of the run and all the spectra were calibrated prior to the identification for obtaining accurate mass values due to the compensation of temperature drift in the mass analyzer. Nine sodium formate clusters were used in the calibration using the high-precision calibration mode.

The accurate mass data for the molecular ions were processed using the software Data Analysis 4.3 to get the list of possible elemental formulae by using SmartFormula. CHNO was used as standard functionalities, including ring-plus double bonds equivalents, electron configuration. Comparison of the theoretical with the measured isotopic pattern was used to obtain mSigma for the suggested molecular formula. The accuracy threshold for most of the compounds is within 25 ppm. A window of ±0.005 to 0.002 *m/z* was used for the extracted ion chromatograms (EIC) to extract the exact masses. Molecular formula determination was carried out by combined evaluation of mass accuracy, isotopic patterns, adduct and fragment information using SmartFormula. Identified compounds were quantified as quercetin equivalents. Different concentrations of quercetin and kaempferol (100, 50, 25, 12.5, 6.25 and 3.125 µg/ml in methanol) were injected (1 µl) into the LC-MS and regression equations were obtained by plotting the peak area of mass spec peak verses concentration.

Statistical Analysis

All results were expressed as means ± SE. The data were evaluated by JMP Pro 12, SPSS and probability of $p < 0.05$ was considered as statistically significant. Triplicate analyses were conducted for each sample.

Results and Discussion

To explore the interaction between bile acids and bioactive compounds in kale, we first aimed to identify the bioactive compounds in kale, then we studied the interaction of bile acids with the Soxhlet-extracted fraction and residue. Finally, we examined the effect of bile acids on the bioaccessibility of kale polyphenols.

Bile Acid Binding Capacity of Solvent Extracts and Fiber-Rich Fractions from Soxhlet Extraction

After Soxhlet extraction, the total dietary fiber, soluble fiber, and insoluble fiber contents of the residue all were higher than in the original raw kale, as expected since the extraction removed most phenolic components of the kale tissue, except the fiber. Lyophilized raw kale had 39.5% total dietary fiber, 6.6% soluble fiber and 32.9% insoluble fiber. By contrast, the Soxhlet-extracted kale residue had 46.6% total dietary fiber, 10.8% soluble fiber, and 35.8% insoluble fiber. After Soxhlet extraction, total dietary fiber increased by 18%, soluble fiber increased by 64%, and insoluble fiber increased by 9% (Table S4). This is due to the loss of compounds extracted by the solvent; 29% of the kale mass was lost during the Soxhlet extraction.

In addition, the fiber composition of the residue was changed by the extraction. The Soxhlet-extracted residue was 71% of the originally loaded kale, and after Soxhlet extraction, total dietary fiber decreased by 16.24%, water-soluble dietary fiber increased by 16.2%, and water-insoluble dietary fiber decreased by 22.7%. This change of soluble fiber and insoluble fiber content was also observed in previous research. For example, Chang's study indicated that heat treatment of apple fiber, corn fiber, oat bran, and soy fiber increased the water-soluble fiber contents and reduced insoluble fiber contents.[82] During Soxhlet extraction, the solvent was evaporated at its boiling temperature, which is a heat treatment to the sample material. This could be the reason why Soxhlet extraction produced an increase in water-soluble dietary fiber and a decrease in water-insoluble dietary fiber. During the Soxhlet extraction, the insoluble dietary fiber may be converted to soluble dietary fiber if the high

temperature breaks the weak bonds between polysaccharide chains and splits glycosidic linkages in the dietary fiber, thus solubilizing the insoluble dietary fiber.[83]

In the experiment that tested the bile acid binding capacity of the Soxhlet-extracted fraction, some sediments appeared. There might be some bile acids trapped or bound by the sediments in the samples. Tests of *in vitro* bile acid binding capacity suggested that the MeOH-extracted fraction, raw kale sample, and Soxhlet-extracted kale residue have similar binding capacities for glycocholate and cholate (See Figure 8). However, the extracted fraction of MeOH: water: formic acid (80:19:1) bound less glycochenodeoxycholate, glycodeoxycholate, chenodeoxycholate and deoxycholate than raw kale and kale residue. Even though the Soxhlet-extracted fraction of MeOH: water: formic acid (80:19:1) bound similar amounts of glycocholate and cholate as raw kale and fiber-rich kale tissue, we found 3.15 mg/g total identified polyphenols in kale (see Section 3.4). Therefore, the polyphenols in kale may not contribute substantially to its bile acid binding capacity.

In the experiment with Soxhlet extracted kale residue, the weight of kale residue after Soxhlet extraction was only 71% of the original weight; therefore, we compared the bile acid binding capacity of 0.71 g kale residue (dry matter) and 1.0 g raw kale (dry matter). The bile acid binding capacity slightly declined after Soxhlet extraction.. Figure 9 compares the bile acid binding capacity of 0.71 g kale residue and 1.0 g raw kale; the results indicated that there is no significant difference between their binding capacities of total bile acids, glycocholic acid, cholic acid, glycochenodeoxycholic acid, chenodeoxycholic acid, and deoxycholic acid. However, the 0.71 g extracted kale residue bound more glycodeoxycholic acid than 1.0 g raw kale. Comparing the bile acid binding capacity of 1.0 g fiber-rich Soxhlet extracted residue and 1.0 g raw kale, the Soxhlet-extracted residue showed higher

binding capacity for total bile acids, glycocholic acid, cholic acid, glycochenodeoxycholic acid, and chenodeoxycholic acid, but similar binding capacity for glycodeoxycholic acid and deoxycholic acid.

Kale is a good source of dietary fiber[84] and dietary fiber has demonstrated bile acid binding capacity through *in vitro* experiments, *in vivo* experiments, and clinical trials.[85-87] Two different hypotheses can explain fiber's ability to bind bile acids. One hypothesis suggested that dietary fiber can physically trap bile acids through hydrophobic or hydrophilic interactions in the gel matrix.[88] The other hypothesis suggested that bile acids can be chemically bound by polysaccharides or dyes, which has a steric moiety.[89]

Our results indicated that 0.71 g extracted residue and 1.0 g raw kale bound similar amounts of glycocholic acid, cholic acid and glycochenodeoxycholic acid. These observations indicate that these three bile acids might be physically trapped by the fiber-rich tissue in kale since the amount of fiber-rich tissue in 0.71 g extracted residue is same with that in 1.0 g raw kale. However, chenodeoxycholic acid may be chemically bound by soluble fiber, since the soluble fiber in 0.71 g Soxhlet extracted residue is 13.6% higher than that in 1.0 g raw kale. Soluble fiber can form a gel, which increases the viscosity in the intestine, and has high bile acid binding capacity.[90] This may be why glycodeoxycholic acid was bound more by 0.71 g kale residue than 1.0 g raw kale. Soluble fiber also shows better hypolipidemic and hypoglycemic responses, compared to insoluble fiber.[90]

By contrast, glycodeoxychholic acid and deoxycholic acid may be chemically bound by insoluble fiber.[89] The insoluble fiber in 0.71 g Soxhlet residue is 22.8% lower than that in 1.0 g raw kale, and 0.71 g Soxhlet residue bound significantly less deoxycholic acid than 1.0 g raw kale. However, the insoluble fiber in 1.0 g Soxhlet extracted residue is only 7.0% higher than that in 1.0 g raw kale, and the residue showed no significant binding

capacity to deoxycholic acid. Camire also reported that deoxycholic acid binding capacity is correlated with insoluble dietary fiber contents.[91]

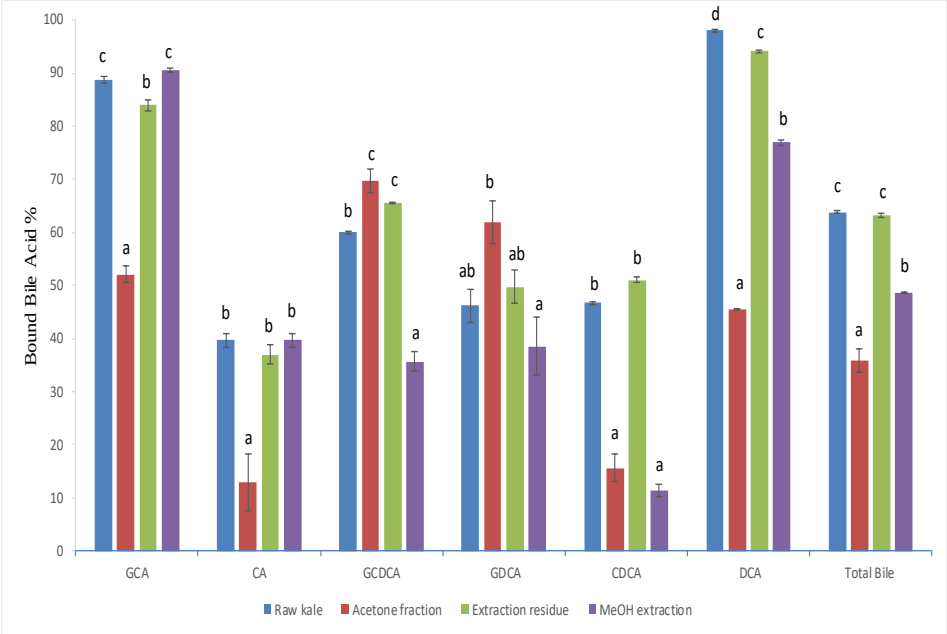

Figure 8. Bile acid binding capacity raw kale, acetone-extracted fraction, extraction residue, methanol extracted fraction. Different letters (a, b, c) indicate that the difference between two values is significant on the level $p<0.05$.

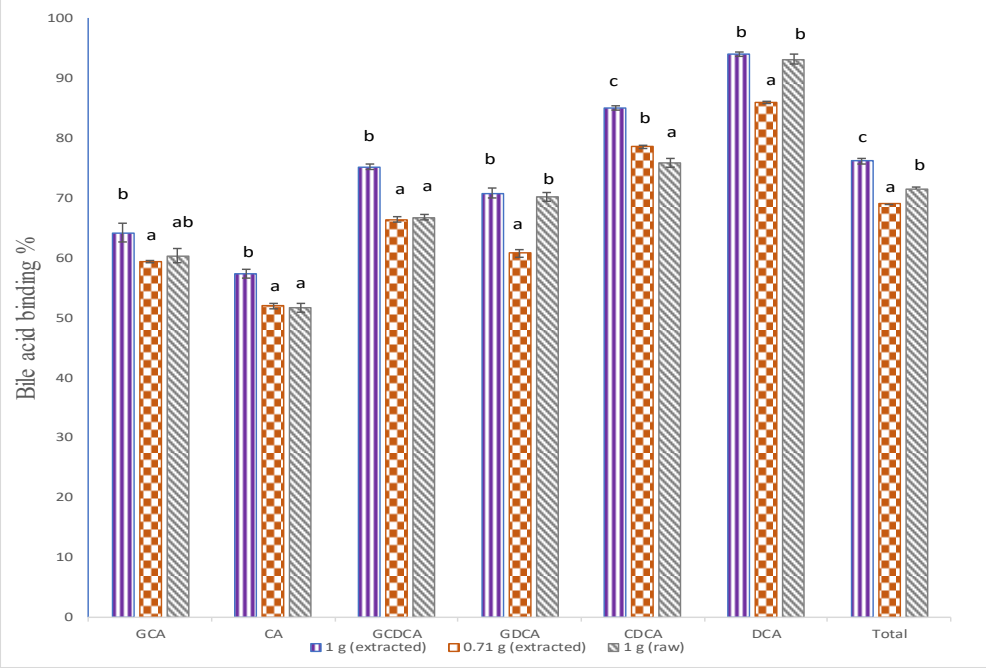

Figure 9. Bile acid binding capacity of raw kale and kale Soxhlet extraction residue. Different letters (a, b, c) indicate that the difference between two values is significant on the level $p<0.05$.

Preliminary Identification and Quantification of Bioactive Compounds in Raw Kale and Incubation with Bile Acids

The tentative identification of phenolic compounds extracted from raw kale with MeOH and MeOH: water: formic acid (80:19:1) is shown in Table 3, which provides information on retention times, protonated molecules, and major fragment ions. Peak identification was conducted based on the data in Table 3, comparing with the compounds in kale from a previously published paper and the standard molecular weights.[22, 33] Compounds from the raw kale include derivatives of kaempferol and quercetin, together with caffeoylquinic acid and sinapoyl derivatives. The UV spectra of various compounds were recorded with a Diode Array Detector (DAD), which indicated that kaempferol derivatives and quercetin derivatives are the main polyphenols presented in kale. Their exact molecular weights cannot be established from LC-MS analysis directly. However, by comparing the molecular ions and fragments obtained with an ESI-MS-MS detector (See Table 3), the compounds were tentatively identified.

We identified nineteen phenolic compounds in the MeOH-extracted fraction, and twenty-seven phenolic compounds in the MeOH: water: formic acid (80:19:1)-extracted fraction. Among these phenolics, we found nine compounds in both the MeOH-extracted and MeOH: water:formic acid (80:19:1)-extracted fractions, including caffeoylquinic acid, three quercetin derivatives, and five kaempferol derivatives. Among the nineteen compounds in the MeOH-extracted fraction, we found seven quercetin derivatives, eleven kaempferol derivatives, and caffeoylquinic acid. The MeOH: water: formic acid-extracted fraction contained nine quercetin derivatives, thirteen kaempferol derivatives, four sinapoyl

derivatives, and caffeoylquinic acid. The kaempferol derivatives are caffeoyl, sinapoyl, feruloyl and hydroxy feruloyl, 3,7-glucosides, diglucoside. The quercetin derivatives are feruloyl, 3,7-glucoside, diglucoside, and triglucoside. The pseudomolecular ions recorded with the HPLC-ESI MS and the fragments obtained confirmed these structures with the characteristic losses of a glucoside, a diglucoside, sinapoyl, caffeoyl and feruloyl residue respectively, leading to the kaempferol aglycone fragment at m/z 287. The high-resolution mass spectrometry analysis confirmed these structures (Table 3).

Kaempferol was used as the molar equivalent to quantify its derivatives, and quercetin was used as the molar equivalent to quantify quercetin derivatives. The results show that quercetin-3-sinapoyl-diglucoside-7-triglucoside is the most abundant phenolic compound extracted from kale in this method, followed by kaempferol-3-(feruloyl)-sophoroside-7-diglucoside, quercetin-3-O-(feruloyl)-sophoroside-7-O-diglucoside, kaempferol-3-sinapoyl-diglucoside-7-O-diglucoside, and quercetin-3-sophoroside-7-glucoside. These are the major polyphenols extracted from green kale in this experiment. Each gram of dry matter kale contained 0.46 mg quercetin-3-sophoroside-7-sophoroside, 0.31 mg quercetin-3-sinapoyl-diglucoside-7-diglucoside, 0.47 mg quercetin-3-sinapyl-diglucoside-7-diglucoside, 0.37 mg quercetin-3-O-(feruloyl)sophoroside-7-O-digulcoside.

The present research found similar compounds to those reported by Ferioli among the polyphenols in kale, such as caffeoylquinic acid, disinapoyl-diglucoside, sinapoyl-feruloyl-diglucoside, trisinapoyl-diglucoside, disinapoyl-feruloyl-gentiobiose.[22] Lin's study showed most of the kaempferol derivatives and quercetin derivatives were the same as in this study.[33] Both kaempferol and quercetin have strong antioxidant capacities[92] and quercetin shows an

inhibitory effect on α-amylase activity, which can help to control blood sugar level in type 2 diabetic patients.[93] The literature indicated that kaempferol and quercetin synergistically inhibit the growth of human intestinal cancer cells.[94] In addition, kale binds bile acids and leaves less bile acid remained for the activation to farnesoid X receptor and G-protein coupled bile acid receptor 1, which are also involved in glucose metabolism, and consequently control blood sugar level in type-2 diabetic patients. Therefore, kale can provide the health benefits of slowing down the ageing process, improving blood sugar control in type-2 diabetic patients, and suppressing intestinal cancer.

The Soxhlet-extracted fraction from raw kale was incubated with bile acids, then analyzed by LC-MS (Table 4). Comparing Table 4 and Table 3 shows that after incubation with bile acids, the polyphenols extracted from kale did not attach to any bile acid to form a new molecule. However, some new polyphenols appeared after incubation with bile acids. These new polyphenols are disinapoyl-diglucoside, 1-sinapoyl-2-feruloyl-diglucoside, 1-sinapoyl-2-feruloyl-gentiobiose, and disinapoyl-feruloyl-gentiobiose. Meanwhile, all six bile acids put into the incubation (glycoholic acid, cholic acid, glycochenodeoxycholic acid, glycodeoxycholic acid, chenodeoxycholic acid, and deoxycholic acid) are still found in the liquid after incubation. Also, we did not find any new molecules formed from the polyphenol attached to the bile acids, which suggested that polyphenols in kale do not contribute to kale's bile acid binding capacity in this *in vitro* experiment. However, Ogawa (2015) reported that some polyphenols, such as catechins and oolongtheanins from tea, can interact

with taurocholic acid and reduce the solubility of phosphatidylcholine (PC) and cholesterol in micelles.[95] Ogawa used nuclear magnetic resonance to show that tea polyphenols and bile acids are in close proximity. Yet the polyphenols and bile acid did not form a new molecule but interacted with each other through hydrogen bonding. This intermolecular force changed the structure of bile acid cholesterol micelles and lowered its solubility. Therefore, according to Ogawa's theory, even though we did not find new molecular forms after incubation of the bile acids with kale polyphenols, it is still possible that polyphenols in kale may interact with bile acids, alter the solubility of bile acids, and influence their reabsorption in the enterohepatic circulation.

Preliminary Identification and Quantification of Bioactive Compounds in Kale of in vitro *Digestion*

After the in vitro digestion (with or without bile acid incubation in small intestine digestion phase), part of these polyphenols existed in raw kale was released into the digestion supernatant, which means they were accessible and eventually could be absorbed by our bodies. Part of the polyphenols remains in the digested residue, indicating that they are not bioaccessible. Table 5 showsthe bioaccessible and non-bioaccessible compounds in digested kale samples, both with and without bile acids. Without bile acids incubation, there are eight compounds released into digestion supernatant, which means they are bioaccessible without bile acids involved in the digestion. Fifteen compounds are found in the digestion residue, when there is no bile acid in the digestive process.With bile acids involved in the *in vitro* digestion, there are twenty-one compounds in the supernatant and sixteen polyphenols in the digested residue. Some compounds are not bioaccessible both with and without bile acids

involved in the digestion process, such as caffeoylquinic acid, kaempferol-3-hydroxyferuloyl-diglucoside-7-O-diglucoside, kaempferol-3-hydroxyferuloyl-diglucoside-7-O-glucoside, kaempferol-3-O-(sinapoyl)-glucoside-7-O-glucoside, kaempferol-3-O-(sinapoyl)-sophoroside-7-glucoside, kaempferol-3-O-(sinapoyl)-sophorotriose, quercetin-3-O-(feruloyl)-sophoroside, 1-sinapoyl-2-feruloyl-diglucoside. As it is shown in Table 5, with bile acids involved in the digestion, fourteen compounds changed from non-bioaccessible to bioaccessible; whereas four compounds were bioaccessible, when no bile acids were in the digestion, and they changed to non-bioaccessible when bile acids are involved in the *in vitro* digestion. They are all kaempferol derivatives. Seventeen phenolic compounds are found in the kale digestion residue with bile acids involved in the digestion process. Among them, only eight polyphenols are found in the digested kale supernatant (see Table 5). The remaining compounds such as quercetin-3-O-sophoroside, kaempferol-3-hydroxyferuloyl-diglucoside-7-diglucoside, kaempferol-3-O-diglucoside-7-O-glucoside, kaempferol-3-O-sophotrioside only appear in the digested residue, indicating that they are not bioaccessible when bile acids are in the digestion. Fifteen phenolic compounds are found in the digested residue without bile acids involved in the digestion, in which ten compounds are only found in the digested residue, indicating that they are not bioaccessible, when bile acids are not involved in the digestive process (see Table 5).

Table 3. Identified polyphenols in raw kale from direct extraction and Soxhlet extraction

RT /min	Compound name	Mol. Formula	Exact mass	ID	Theratical Value	Mass error (ppm)	MS/MS fragment	Soxhlet extraction	Direct extraction
15.4	Caffeoylqunic acid	C16H18O9	731.1950	$[2M+Na]^+$	731.1799	20.6064	377, 215, 163	X	X
20	Quercetin-3-hydroxferuloyl-diglucoside-7-glucoside	C43H48O26	981.2325	$[M+H]^+$	981.2507	-18.5050	795, 633, 449, 287		X
20.2	Quercetin-3-O (caffeoyl)-sophoroside-7-glucoside	C39H50O27	951.2249	$[M+H]^+$	951.2612	-38.1946	795, 571, 303, 163		X
21.5	Quercetin-3-sinapyl-diglucoside-7-triglucoside	C50H60O31	995.2490	$[M+H]^+$	995.2663	-17.3903	553, 465, 369, 303, 207, 175		X
22.1	Quercetin-3-O-(feruloyl)-sophoroside-7-O-digulcoside (isomer)	C49H58O29	1111.2990	$[M+H]^+$	1111.3137	-13.1844	523, 449, 339, 287, 177, 145, 117		X
22.8	Quercetin-3-diglucoside-7-glucoside	C33H40O22	789.2250	$[M+H]^+$	789.2084	21.0350	649, 465, 303	X	X
23.7	Quercetin-3-diglucoside-7-glucoside (isomer)	C33H40O22	789.2230	$[M+H]^+$	789.2084	18.5008	649, 541, 465, 407, 303	X	X
23.9	Quercetin-3-sophoroside-7-diglucoside	C39H50O27	951.2790	$[M+H]^+$	951.2612	18.6773	649, 465, 347, 303	X	X
24.2	Quercetin-3-sophoroside-7-glucoside	C33H40O22	789.1691	$[M+H]^+$	789.2084	-49.7955	705, 633, 492, 449, 411, 339, 303,		X
26.1	Quercetin-3-O-(feruloyl)-sophoroside	C43H48O25	965.2477	$[M+H]^+$	965.2557	-8.3325	909, 825, 685, 571, 523, 411, 339, 303, 251, 177, 145, 89		X
26.9	Quercetin- 3- feruloyl-diglucoside-7-glucoside	C43H48O25	965.2670	$[M+Na]^+$	965.2557	11.6622	825, 539, 465, 339, 303, 177	X	
27.1	Quercetin-3-sinapoyl-diglucoside-7-diglucoside	C50H60O31	1157.3330	$[M+H]^+$	1157.3191	11.9829	855, 553, 465, 369, 303, 207	X	X
27.5	Quercetin-3-feruloyl-diglucoside-7-diglucoside	C49H58O30	1127.3190	$[M+H]^+$	1127.3086	9.2548	825, 523, 465, 339, 303, 177	X	X
28.6	Quercetin-3-disinapoyl-triglucoside-7-diglucoside	C67H80O40	1525.4132	$[M+H]^+$	1525.4299	-10.9241	912, 737, 539, 457, 393, 303, 207, 175, 91		X
29.9	Quercetin-3-glucoside-7-O-glucoside	C27H30O17	627.1600	$[M+H]^+$	627.1556	7.0541	303	X	
20.5	Kaempferol-3-O-sophotrioside-7-O-glucoside	C42H46O24	957.2293	$[M+Na]^+$	957.2277	1.6966	633, 571, 439, 347, 287, 193		X
20.8	Kaempferol-3-O-(methoxycaffeoyl)sophoroside-7-O-glucoside	C43H48O25	965.2390	$[M+Na]^+$	965.2557	-17.3457	539, 449, 355, 337, 287, 193, 161, 133, 105		X
21.2	Kaempferol-3-hydroxyferuloyl-diglucoside-7-diglucoside	C49H58O30	1127.2869	$[M+H]^+$	1127.3086	-19.2201	957, 539, 449, 355, 287, 193, 133, 89		X
23.9	Kaempferol-3-O-sophotoside-7-O-diglucoside	C42H46O24	957.2062	$[M+Na]^+$	957.2277	-22.4356	649, 561, 449, 411, 347, 287, 160, 117		X
24.1	Kaempferol-3-O-diglucoside-7-glucoside	C33H40O21	773.2150	$[M+H]^+$	773.2135	1.9594	633, 449, 287	X	
25.4	Kaempferol – 3- hydroxyferuloyl-diglucoside-7-glucoside	C43H48O25	965.2710	$[M+Na]^+$	965.2557	15.8062	825, 633, 539, 449, 355, 287, 193	X	

Continue Table 3

25.9	Kaempferol-3-O-(caffeoyl)sophoroside-7-O-glucoside	C42H46O24	935.2590	$[M+H]^+$	935.2663	-7.8138	795, 509, 449, 287, 163	X	
26.1	Kaempferol-3-hydroxyferuloyl-diglucoside-7-diglucoside	C49H58O30	1127.3250	$[M+H]^+$	1127.3086	14.5772	957, 825, 539, 449, 355, 287, 193	X	
26.7	Kaempferol-3-O-(caffeoyl)-sophoroside-7-O-diglucoside	C45H60O31	1097.3130	$[M+H]^+$	1097.3191	-5.5882	1017, 855, 795, 553, 409, 287	X	X
26.8	Kaempferol-3-O-(sinapoyl)-sophoroside	C38H40O20	817.2058	$[M+H]^+$	817.2186	-15.6262	668, 593, 553, 523, 453, 411, 369, 287		X
27.9	Kaempferol-3-O-(feruoyl)-sophoroside	C37H38O20	803.1894	$[M+H]^+$	803.2029	-16.8326	523, 457, 409, 339, 287, 177,145, 89		X
28	Kaempferol-3-O-(feruloyl)-sophoroside	C37H38O19	787.2044	$[M+H]^+$	787.2080	-4.5795	619, 538, 442, 339, 287, 236, 176, 105		X
28.2	Kaempferol-3-sinapoyl-diglucoside-7-glucoside	C44H50O25	979.280	$[M+Na]^+$	979.2714	8.7882	839, 553, 449, 369, 287, 207.	X	
28.4	Kaempferol-3-sinapoyl-diglucoside-7-diglucoside	C50H60O30	1141.3310	$[M+H]^+$	1141.3242	5.9431	1001, 839, 633, 553, 449, 369, 287, 207	X	X
28.7	Kaempferol-3-(feruoyl)sophoroside-7-O-glucoside	C43H48O24	949.2680	$[M+H]^+$	949.2608	7.5543	809, 707, 523, 449, 339, 287, 177	X	
29.1	Kaempferol-3-feruloyl-digulcoside-7-digucoside	C49H58O29	1111.3320	$[M+H]^+$	1111.3137	16.5102	809, 523, 435, 339, 287, 177	X	X
29.2	Kaempferol-3-disinapoyl-triglucoside-7-diglucoside	C67H80O39	1509.4099	$[M+H]^+$	1509.4349	-16.5950	1115, 891, 737, 369, 287, 207, 175, 91		X
30.9	Kaempferol-3-O-sophoroside	C27H30O16	611.1650	$[M+H]^+$	611.1607	7.0996	287	X	
31.1	Disinapoyl-diglucoside	C34H42O19	777.2083	$[M+Na]^+$	777.2218	-17.3670	603, 553, 531, 479, 393, 369, 287, 207, 145, 91		X
31.3	1-sinapoyl-2-feruloyl-diglucoside	C33H40O18	747.1982	$[M+Na]^+$	747.2112	-17.4422	603, 523, 339, 287, 207, 177, 145, 89		X
32.9	Trisinapoyl-diglucoside	C45H52O23	983.2719	$[M+Na]^+$	983.2797	-7.9398	759, 369, 285, 243, 207, 175, 91		X
33.2	Disinapoyl-feruloyl-gentiobiose	C44H50O22	953.2670	$[M+Na]^+$	953.2691	-2.2470	729, 535, 369, 207, 177, 91		X

Table 4. Identified polyphenols in Soxhlet extracted fraction after incubation with bile acids

RT/min	Tentative identification	MS (m/z);	ID	MS ions (m/z of the main fragments)	Formula
15.7	Caffeoylquinic acid	377.2223	$[M+Na]^+$	264, 235, 163, 135, 117, 89	C16H18O9
16.9	p-coumaroylquinic acid	699.1847	$[2M+Na]^+$	511, 361, 147, 119, 91	C16H18O8
23.5	Quercetin-3-sophoroside-7-glucoside	789.504	$[M+H]^+$	649, 541, 347, 303	C33H40O22
24.6	Quercetin-3-sophoroside-7-diglucoside	951.6187	$[M+H]^+$	795, 649, 465, 347, 303	C39H50O27
24.9	Kaempferol-3-O-sophoroside-7-glucoside	773.5072	$[M+H]^+$	633, 571, 449, 347, 287	C36H36O19
25.9	Kaempferol-3-O-(caffeoyl)sophoroside-7-O-glucoside	957.6164	$[M+Na]^+$	633, 449, 347, 287, 163	C42H46O24
26.3	Kaempferol – 3- hydroxyferuloyl-diglucoside-7-glucoside	987.6139	$[M+Na]^+$	825, 539, 449, 355, 287, 193, 133	C43H48O25
26.9	Kaempferol-3-O-(caffeoyl)sophoroside-7-O-glucoside	957.5905	$[M+Na]^+$	795, 509, 449, 287, 163, 135	C42H46O24
27.1	Quercetin-3-feruloyl-diglucoside-7-diglucoside	1127.7371	$[M+H]^+$	957, 825, 539, 449, 287, 193, 84	C49H58O30
27.7	Kaempferol-3-O-sophorotrioside-7-O-diglucoside	1097.7141	$[M+H]^+$	1017, 795, 553, 287	C45H60O31
28.1	Quercetin-3-O-sinapoyl-diglucoside-7-diglucoside	1179.7539	$[M+Na]^+$	987, 855, 553, 369, 303, 207, 175	C50H60O31
28.6	Quercetin-3-feruloyl-diglucoside-7-diglucoside	1127.7337	$[M+H]^+$	825, 523, 465, 339, 303, 177	C49H58O30
29.3	Kaempferol-3-sinapoyl-diglucoside-7-glucoside	979.6311	$[M+H]^+$	839, 633, 553, 449, 369, 287, 207	C44H50O25
29.8	Kaempferol-3-sinapoyl-diglucoside-7-diglucoside	1141.7498	$[M+H]^+$	971, 809, 707, 523, 435, 287, 177	C50H60O30
30.2	Kaempferol-3-feruloyl-digulcoside-7-digucoside	1111.7294	$[M+H]^+$	809, 523, 339, 287, 177	C49H58O29
23.3	Glycoholic acid	1396.927	$[3M+H]^+$	931, 488, 412, 337, 209, 91	C26H43NO6
24.9	Cholic acid	1225.8692	$[3M+H]^+$	817, 431, 355, 239, 105	C24H40O5
25.3	Glycochenodexoycholic acid	1348.9548	$[3M+H]^+$	899, 414, 339, 91	C26H43NO5
25.7	Glycodexoycholic acid	1348.9505	$[3M+H]^+$	899, 450, 339	C26H43NO5
27.6	Chenodeoxycholic acid	1177.8885	$[3M+H]^+$	785, 357	C24H40O4
28.1	Deoxycholic acid	1177.8878	$[3M+H]^+$	823, 785, 415, 357, 247	C24H40O5

Bioaccessibility of Phenolic Compounds in Kale

Kaempferol was used as a molar equivalent to quantify kaempferol derivatives and quercetin was used as a molar equivalent to quantify quercetin derivatives. The preliminarily identified polyphenols in raw kale are shown in Table 3. In raw kale, the total quantified polyphenols were 3.01 mg/g and included 1.98 mg quercetin derivatives and 1.03 mg kaempferol derivatives. The tentatively quantified bioaccessible compounds are shown in Table 3. These are the compounds released into the supernatant after *in vitro* digestion, either with or without bile acids in the digestion. With bile acids in the digestion, a total of 2.09 mg/g polyphenols was released into the supernatant, including 1.38 mg quercetin derivatives and 0.71 mg kaempferol derivatives. For the polyphenols in raw kale, with bile acids in the digestion, 69.4% of polyphenols were bioaccessible, 69.5% of quercetin derivatives were bioaccessible, and 69.1% of kaempferol derivatives were bioaccessible. With no bile acids in the digestion, we found 0.44 mg/g of polyphenols, and 0.05 mg of quercetin derivatives were bioaccessible and 0.39 mg of kaempferol derivatives were bioaccessible. Therefore, when no bile acids were in the *in vitro* digestion, 14.6% of identified polyphenols in kale were bioaccessible, and 37.7% kaempferol derivatives were bioaccessible, but only 2.7% of quercetin derivatives were bioaccessible.

In the residue of the digestion with bile acids, 0.37 mg/g polyphenols remain biostable, which means these polyphenols stay in the digested residue and not released into the digestive system. Among them, 0.18 mg are quercetin derivatives and 0.19 mg/g are kaempferol derivatives. Therefore, as is shown in Figure 10, with bile acids involved in the digestion, the biostability of quercetin derivatives was 9.2%, the biostability of kaempferol

derivatives was 18.0%, and 12.2% of total identified polyphenols were biostable. In the residue without bile acids, 0.37 mg/g of polyphenols remained biostable, among them 009 mg/g quercetin derivatives and 0.14 mg/g kaempferol derivatives. Hence, when no bile acids were in the digestion, the biostability of quercetin derivatives is 4.2%, biostability of kaempferol derivatives is 12.0%, totally 6.8% identified polyphenols are biostable, as is suggested Figure 10.

Therefore, bile acids involved in the digestion can significantly improve the bioaccessibility of total identified polyphenols and quercetin derivatives. However, the bioaccessibility of kaempferol derivatives is slightly higher in the samples without bile acids in the digestion. This phenomenon probably is associated with the dietary fiber in kale. When the kale enters the human digestive system, our mouth and stomach work like an extractor for the polyphenols in kale. In our mouth, the kale is broken down mechanically; in our stomach, the acid helps to extract polyphenols in the vacuoles and weaken the link between polyphenols and the fiber. However, in the small intestine, the dietary fiber may act as an entrapping matrix to restrict the diffusion of polyphenols and limit their bioaccessibility.[12] Without bile acids in the digestion, dietary fiber can trap more polyphenols; when bile acids are added into the *in vitro* digestion, dietary fiber can bind more bile acids, which frees polyphenols and thus elevates the bioaccessibility of polyphenols.

Table 5. Phenolic compounds in kale from digestion supernatant (with/without bile acids incubation), and digestion residue (with/without bile acids incubation).

RT [min]	Description	Raw Kale (mg/g)	Supernatant without bile acids (mg/g)	Supernatant with bile acids (mg/g)	Residue without bile acids (mg/g)	Residue with bile acids (mg/g)
18.5	Quercetin-3-sophoroside-7-sophoroside	ND	0.025 ± 0.002	0.051 ± 0.001	0.026 ± 0.009	0.025
19.1	Quercetin-3-sophoroside-7-glucoside	0.128 ± 0.016	ND	0.285 ±0.005	0.016 ± 0.002	0.053 ± 0.001
19.9	Quercetin-3-triglucoside-7-glucoside	0.421 ± 0.018	ND	0.054 ± 0.001	ND	ND
19.7	Quercetin-3-sophororose-7-O-glucoside	0.087 ± 0.005	ND	ND	ND	ND
20.1	Quercetin-3-hydroxferuloyl-diglucoside-7-glucoside	0.051 ± 0.004	ND	ND	ND	ND
20.2	Quercetin-3-O-(caffeoyl)-sophoroside-7-O-glucoside	0.041 ± 0.003	ND	0.040 ± 0.010	ND	ND
21	Quercetin-3-O-(feruloyl)-sophoroside-7-O-glucoside (isomer)	ND	ND	0.387 ± 0.016	ND	ND
21.5	Quercetin-3-O-sophoroside	ND	ND	ND	ND	0.030 ± 0.001
21.5	Quercetin-3-sinapoyl-diglucoside-7-glucoside	0.150 ± 0.013	ND	ND	ND	0.016
21.6	Quercetin-3-sinapyl-diglucoside-7-diglucoside	0.520 ± 0.027	ND	0.235 ± 0.014	ND	ND
21.8	Quercetin-3-O-(feruloyl)-sophoroside-7-O-digulcoside	0.455 ± 0.032	0.028 ± 0.007	0.140 ± 0.010	0.024 ± 0.002	0.024
22.2	Quercetin-3-glucoside-7-glucoside	ND	ND	ND	0.016 ± 0.001	ND
24.2	Quercetin-3-O-(feruloyl)-sophoroside-7-O-glucoside	ND	ND	0.020 ± 0.005	ND	0.041 ± 0.001
24.4	Quercetin-3-O-sinapoyl-diglucoside-7-O-glucoside	ND	ND	0.037 ± 0.001	ND	ND
26	Quercetin-3-O-(feruloyl)-sophoroside	0.027 ± 0.002	ND	0.079	ND	0.030 ± 0.001
28.6	Quercetin-3-disinapoyl-triglucoside-7-diglucoside	0.103 ± 0.004	ND	0.049 ± 0.002	ND	ND
16.9	Kaempferol-3-O-glucoside	ND	ND	ND	0.004	ND
19.7	Kaempferol-3-O-diglucoside-7-O-glucoside	ND	0.074 ± 0.001	ND	ND	0.009 ± 0.002
20.3	Kaempferol-3-O-diglucoside-7-O-diglucoside	ND	0.026 ± 0.001	0.014	0.007 ± 0.001	ND

Continue Table 5

20.3	Kaempferol-3-O-sophotrioside-7-O-glucoside	0.014 ± 0.001	ND	ND	ND	ND
20.7	Kaempferol-3-O-(hydroxyferuloyl)-sophoroside-7-O-glucoside	0.157 ± 0.005	ND	0.040 ± 0.002	ND	0.014
21.2	Kaempferol-3-O-(caffeoyl)-sophoroside-7-O-diglucoside	0.019 ± 0.002	ND	ND	ND	ND
21.8	Kaempferol-3-hydroxyferuloyl-diglucoside-7-diglucoside	0.123 ± 0.004	ND	ND	ND	0.014
21.5	Kaempferol-3-O-sophotrioside	ND	0.018 ± 0.002	0.044 ± 0.001	ND	0.012
22.1	Kaempferol-3-O-sophotrioside (isomer)	ND	ND	ND	ND	0.041 ± 0.001
22.3	Kaempferol-3-sinapoyl-diglucoside-7-O-digulcoside	0.311 ± 0.006	ND	0.214 ± 0.007	0.009 ± 0.002	ND
22.4	Kaempferol-3-feruloyl-digulcoside-7-digucoside	0.327 ± 0.010	0.171 ± 0.007	0.199 ± 0.005	0.008	0.038 ± 0.001
23.1	Kaempferol-3-O-glucoside-7-O-glucoside	ND	ND	ND	0.007 ± 0.001	ND
23.6	Kaempferol-3-(feruoyl)-sophoroside-7-glucoside	ND	ND	ND	0.006 ± 0.001	ND
24.2	Kaempferol-3-O-diglucoside	ND	ND	ND	0.018 ± 0.002	ND
24.7	Kaempferol-3-(feruoyl)-sophorotriose	ND	0.045 ± 0.002	ND	0.042 ± 0.004	ND
25	Kaempferol-3-(feruoyl)-sophoroside-7-diglucoside (isomer)	ND	ND	0.054 ± 0.002	0.018 ± 0.006	0.017 ± 0.001
25.3	Kaempferol-3-O-(sinapoyl)-sophorotriose	ND	ND	0.032 ± 0.001	ND	0.011
26.8	Kaempferol-3-O-(sinapoyl)-glucoside-7-glucoside	ND	ND	0.033 ± 0.001	ND	ND
26.8	Kaempferol-3-O-(sinapoyl)-sophoroside	0.013	ND	ND	ND	0.012
27.3	Kaempferol-3-O-(methoxycaffeoyl)-diglucoside	ND	ND	ND	0.006 ± 0.001	ND
27.4	Kaempferol-3-O-(feruoyl)-sophoroside	0.015 ± 0.001	0.051 ± 0.002	ND	0.022 ± 0.003	0.018
27.7	Kaempferol-3-O-(feruloyl)-glucoside-7-glucoside	ND	ND	0.055 ± 0.002	0.004	ND
28.4	Kaempferol-3-(feruoyl)-sophoroside-7-diglucoside (isomer)	ND	ND	0.007 ± 0.001	ND	ND
29.2	Kaempferol-3-disinapoyl-triglucoside-7-diglucoside	0.035 ± 0.003	ND	0.017 ± 0.001	ND	ND
31.1	Kaempferol-3-O-(feruloyl)-sophoroside (isomer)	0.011 ± 0.001	ND	ND	ND	ND

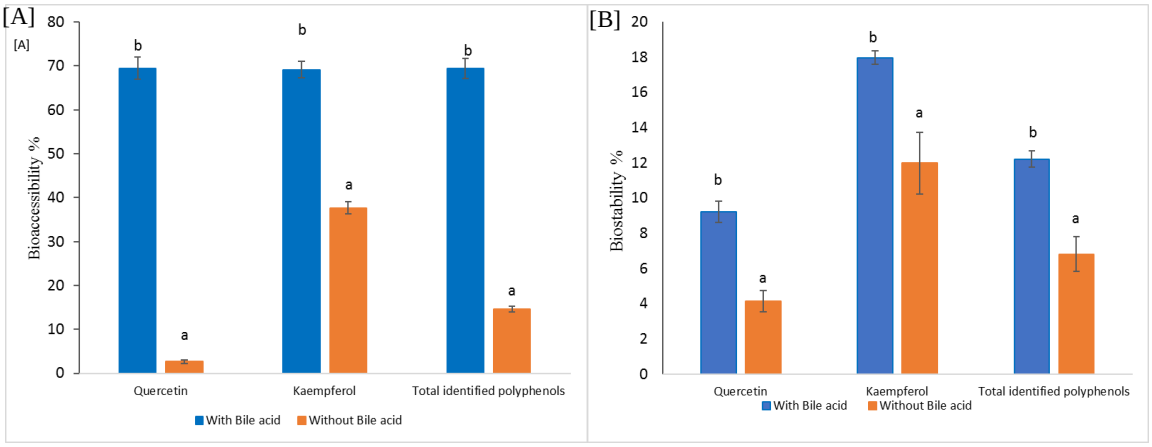

Figure 10. Bioaccessibility (A) and biostability (B) of kaempferol derivatives, quercetin derivatives and total identified polyphenols after the *in vitro* digestion with / without bile acids.

Conclusion

After Soxhlet extraction of kale, the residue was 71% of the original weight and after Soxhlet extraction, total dietary fiber increased 18%, soluble fiber increased 64%, and insoluble fiber increased 9%. However, 0.71 g of the residue showed similar binding capacity to that of 1.0 g raw kale for glycocholate, cholate, glycochenodeoxycholate, chenodeoxycholate, and deoxycholate. Yet, 0.71 g of extraction residue bound more glycodeoxycholate than 1.0 g of raw kale. This suggested that the main part in kale that contributes to bile acid binding is the fiber-rich tissue. Polyphenols in kale are quercetin derivatives, kaempferol derivatives, and caffeoylquinic acid acid. These polyphenols do not bind bile acids, but may interact with bile acids through intermolecular force. Bile acids in the digestion significantly increase the bioaccessibility of quercetin derivatives, and total identified polyphenols, but slightly reduced the bioaccessibility of kaempferol derivatives.

CHAPTER V

SUMMARY

Bile acids assist in the digestion of fatty foods, are reabsorbed through the ileum, and can be transferred back to the liver via the enterohepatic circulation. Binding of bile acids by foods can reduce bile acid reabsorption and promote synthesis of new bile acids from cholesterol, which can lower cholesterol levels and prevent cardiovascular disease. The bile acid binding capacity of red kale, green kale, red leaf lettuce, Brussels sprouts, and red cabbage were examined through *in vitro* digestion. These vegetables' binding capacity for total and individual bile acids was analyzed by HPLC. The results showed that green kale and red kale have higher *in vitro* bile acid binding capacity than the rest of the vegetables. Also, kale preferentially binds hydrophobic bile acids, deoxycholic acid, and chenodeoxycholic acid. However, green kale was chosen for further study, since it has more phenolic compounds than red kale.

Different bile acid compositions were used to test green kale's bile acid binding capacity. Kale shows similar binding capacity in the mixture mimicking the bile acid composition of a male with gallstones and a healthy female, but it binds fewer bile acids in the composition simulating the bile of a male with type-2 diabetes. In the type-2 diabetic male's bile acid composition, green kale can bind no more than 81.8% of total bile acids and theoptimal dose for this maximal binding capacity is 1.31 g dry weight in this experiment. Considering this result and the data from previous clinical trials,[57, 96] green kale can bind no more than 51.6% of bile acids in the human body, which would need 55.6 g green kale (dry matter) or 427.0 g fresh kale. Considering that 427.0 g fresh kale is likely too much for daily consumption, lyophilized kale snacks may be a better choice. However, most of the kale

chips in the market are fried and flavored with sugar, which may cause additional problem to hypercholesterolemia it is wiser to choose freeze dried kale snacks.

Microwave heating of kale for 1 or 3 min can significantly improve kale's *in vitro* bile acid binding capacity. This may be because heating changed the structure of the protein and dietary fiber complex. Dietary fiber can bind bile acids, and ample evidence[30, 85, 97] indicates that when protein is separated from the dietary fiber complex, this increases the available surface of the fiber for more bile acid binding.[31] However, dietary fiber from different vegetables shows different bile acid binding capacities. For example, each gram of dietary fiber from red cabbage can bind 84.2 mg of bile acids, but each gram of dietary fiber from red leaf lettuce can bind 109.2 mg of bile acids. Other compounds in the vegetables might increase the bile acid binding capacity of the dietary fiber.

To better understand the interaction of other bioactive compounds with bile acids, kale was used for further study. Lyophilized kale was extracted with a Soxhlet apparatus, and the polyphenols were separated from the dietary fiber. The polyphenols were characterized by ultra-high-performance liquid chromatography-time-of-flight-mass spectrometry (LC-QTOF-MS). Twenty-seven polyphenols were found in the raw kale, including nine quercetin derivatives, thirteen kaempferol derivatives, four sinapoyl derivatives, and caffeoylquinic acid.

To test whether polyphenols contributed to bile acid binding, the fiber-rich tissue and extracted phenolic compounds were incubated with bile acids. The results indicated that bile acids are mainly bound by the fiber-rich tissue, rather than by phenolic compounds. Polyphenols attaching to bile acids can form novel molecules. However, the polyphenols may still interact with bile acids through intermolecular force and contribute to bile acid

binding. In addition, bile acids in the digestive process can improve the bioaccessibility of polyphenols, which mayoccur when bile acids are bound to the dietary fiber in the kale, because dietary fiber released polyphenols and increased their bioaccessibility.

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APPENDIX A

Table S1. Chemical composition of simulated saliva fluid prepared for the bile acid binding assay[53]

Chemicals	(g/l)
Sodium chloride	1.594
Ammonium nitrate	0.328
Potassium dihydrogen phosphate	0.636
Potassium chloride	0.202
Potassium citrate	0.308
Uric acid sodium salt	0.198
Lactic acid sodium salt	0.146
Porcine gastric mucin	1.000

Table S2. Different bile acids compositions used in the present study to mimic the various human health conditions[98]

Bile acid	BAC-1 (mM) (%)	BAC-2 (mM) (%)	BAC-3 (mM) (%)
Glycocholate	1.24 (3.1)	1.2 (3.1)	1.24 (3.1)
Cholate	12.1 (30.2)	12 (30.0)	13.2 (30.2)
Glycochenodeoxycholate	5.1 (12.8)	2.56 (6.4)	5.1 (12.8)
Glycodeoxycholate	2.5 (6.3)	2.5 (6.3)	2.6 (6.3)
Chenodeoxycholate	11.6 (29.0)	17.4 (43.5)	5.8 (14.5)
Deoxycholate	7.4 (18.6)	4.28 (10.7)	13.7 (33.0)

BAC-1: male with gallstone

BAC-2: healthy female

BAC-3: type 2 diabetes

Table S3. Moisture content in fresh vegetables, total fiber, and lyophilized amount used for bile acid binding assay

Vegetables	Moisture (%)	Dried sample used for bile acid binding (g)	Total fiber (%)	Equivalent to fresh weight (g)	Total bile acid binding (%)
Red kale	87.29	2	40	15.74	89.73
Red leaf lettuce	95.64	2	31.5	45.87	78.06
Green kale total	89.08	2	39.5	18.32	86.5
Green kale leaf	86.98	2	38.1	15.36	81.40
Brussels sprouts	84.36	2	30.4	12.79	62.09
Red cabbage	91.29	2	30.3	22.96	57.91

Table S4. Fiber content of lyophilized green kale and Soxhlet extracted green kale residue

Sample	Total dietary fiber %	Soluble fiber %	Insoluble fiber %
Lyophilized Green Kale	39.5	6.6	32.9
Soxhlet extracted residue of Green kale	46.6	10.8	35.8
71% Soxhlet extracted residue of Green Kale	33.1	7.5	25.4