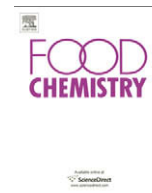
Food Chemistry 119 (2010) 592–599

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Inhibitory effect of raspberries on starch digestive enzyme and their antioxidant properties and phenolic composition

Lei Zhang [a], Jianrong Li [a], Shelly Hogan [b], Hyun Chung [b], Gregory E. Welbaum [c], Kequan Zhou [d,*]

[a] *College of Food Science, Biotechnology and Environmental Engineering, Zhejiang Gongshang University, Hangzhou 310035, China*
[b] *Department of Food Science and Technology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA*
[c] *Department of Horticulture, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, USA*
[d] *Department of Nutrition and Food Science, Wayne State University, 3003 Science Hall, Detroit, MI 48202, USA*

ARTICLE INFO

Article history:
Received 5 February 2009
Received in revised form 24 June 2009
Accepted 30 June 2009

Keywords:
Raspberry
Digestive enzymes
α-Glucosidase
ORAC
Anthocyanins
Phenolic acids

ABSTRACT

Seven primocane fall-bearing raspberry (*Rubus idaeus* L.) cultivars, Nova (red), Dinkum (red), Heritage (red), Autumn Britten (red), Josephine, Anne (yellow), Fall Gold (yellow) were analysed for potential health promoting properties including their inhibitory effect on starch and fat digestive enzymes, antioxidant activities, and phenolic composition. The tested raspberry extracts showed no detectable inhibition of pancreatic α-amylase and lipase. However, all the extracts exhibited potent inhibition of α-glucosidase with IC_{50} from 16.8 to 34.2 μg/mL. Four phenolic compounds, ellagic acid, cyanidin-diglucoside, pelargonidin-3-rutinoside, and catechin were identified as the active α-glucosidase inhibitors. The raspberry extracts also possessed significant antioxidant activities with oxygen radical absorbance capacities (ORAC) ranging from 136.7 to 205.2 μmol Trolox equivalents (TE)/g dry weight fruit and DPPH radical scavenging activities from 305 to 351 μmol TE/g. The total phenolic content of raspberry cultivars varied significantly from 40.9 to 98.5 mg of gallic acid equivalents/g dry weight. The anthocyanin content varied widely from 0.1 to 9.5 mg cyanidin 3-glucoside equivalents/g. Nine phenolic acids were quantified in raspberries and their total amounts varied from 157.3 to 713.5 μg/g. The enzyme inhibition and antioxidant properties of raspberry cultivars were not correlated with their total phenolic, anthocyanin, and phenolic acid content. Overall, 'Dinkum' and 'Josephine' raspberry varieties possess higher total phenolic content, ORAC, DPPH radical scavenging activity, and α-glucosidase inhibitory activity than other five cultivars.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

A growing body of epidemiological and clinical evidence suggests that sufficient consumption of fruits is associated with a wide variety of health benefits such as reduced risk of cardiovascular disease, diabetes, and certain types of cancers (Estaquio et al., 2008; Villegas, Salim, Flynn, & Perry, 2004; Weisburger, 2002). Fruits contain numerous bioactive components and are especially rich in phenolic compounds such as flavonoids, phenolic acids, stilbenes, and procyanidins (Naczk & Shahidi, 2006). These compounds are believed to work synergistically to promote human health through a variety of different mechanisms, such as enhancing antioxidant activity, impacting cellular processes associated with apoptosis, platelet aggregation, blood vessel dilation, and enzyme activities associated with carcinogen activation and detoxification (Shahidi & Wanasundara, 1992; Stevenson & Hurst, 2007; Zafra-Stone et al., 2007). Elucidating the full potential of the health promoting capabilities of fruits continues to enhance and advance the discipline of functional foods and nutraceutical research.

Raspberries (*Rubus idaeus* L.) are popular aggregate fruits from the family Rosaceae. The fruits are known as a rich source of dietary antioxidants largely due to their high level of phenolic compounds, which are primarily comprised of cyanidins, anthocyanins, ellagitannins, phenolic acids, and conjugates of ellagic acid and quercetin (Beekwilder, Hall, & de Vos, 2005; Mullen et al., 2002). A recent study reported that a raspberry extract has the highest cellular antioxidant activity among 25 fruits commonly consumed in the United States (Wolfe et al., 2008). In addition to strong antioxidant properties, raspberries have also shown other beneficial bioactivities including anti-inflammation, antimicrobial activity against pathogenic intestinal bacteria, and anti-proliferation of human liver ($HepG_2$), breast (MCF-7), colon (HT-29, HCT116), and prostate (LNCaP) cancer cells (Liu et al., 2002b; Seeram et al., 2006).

More recently, the extract of a specific raspberry (Glen Ample) was found to strongly inhibit activities of starch digesting enzymes α-glucosidase and α-amylase (McDougall & Stewart, 2005). The inhibition of these enzymes reduces starch digestion and

* Corresponding author. Tel.: +1 313 577 5566; fax: +1 313 577 8616.
E-mail address: kzhou@wayne.edu (K. Zhou).

0308-8146/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2009.06.063

absorption, consequently lowering a postprandial hyperglycemic response. This hypoglycemic effect is an established and effective target for type 2 diabetes prevention and treatment (Chiasson et al., 2002; Toeller, 1994; van de Laar et al., 2005). The phenolic compounds in raspberry may play a key role in the inhibition of starch digesting enzymes. Studies have shown that one particular group of phenolic compounds, diacylated anthocyanins, are effective α-glucosidase inhibitors and able to induce an anti-hyperglycemic response in diabetes animal models (Matsui et al., 2001; Matsui et al., 2004). Furthermore, proanthocyanidins, another group of phenolic compounds, have also been detected to inhibit activities of digestive enzymes (Yuste, Longstaff, & McCorquodale, 1992). The inhibition of starch digestive enzymes by dietary phenolics may represent a biochemical rationale or mechanism for delivering some of the health benefits attributed to a diet rich in fruits.

Raspberries belong to a diverse group of species and hybrids in the genus *Rubus*, therefore, it is highly likely that their phytochemical composition and associated bioactive properties may vary due to this genetic diversity. For instance, Beekwilder et al. showed that the concentrations of the major components in 14 raspberry cultivars varied as much as 20-fold (Beekwilder, Hall et al., 2005; Beekwilder, Jonker et al., 2005). Currently, little is known about the effects of different raspberries on starch digestive enzymes (McDougall, Ross, Ikeji, & Stewart, 2008). As such, the aim of this study was to assess the inhibitory activities of different raspberry cultivars with diverse genetic backgrounds against α-glucosidase and α-amylase in an effort to identify specific cultivars and active constituents with potent enzyme inhibitory activities. Determining the inhibitory profile of raspberry phenolics against carbohydrate modulating enzymes related to glucose absorption in the intestine may demonstrate its potential for managing blood glucose levels in populations with or at risk for type 2 diabetes. Other bioactivities relevant to diabetes, including antioxidant properties, were also evaluated among raspberry cultivars. In addition, we examined the effect of the raspberries on lipid digestive enzyme (pancreatic lipase) for comparison. The quantity of the major phenolic compounds in different raspberries was also determined.

2. Material and methods

2.1. Chemicals and reagents

Porcine pancreatic α-amylase (EC 3.2.1.1), yeast α-glucosidase (EC 3.2.1.20), porcine pancreatic lipase (EC 3.1.1.3), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-bipyridyl, disodium ethylenediaminetetraacetatic acid (EDTA), fluorescein, and phenolic acid standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anthocyanin standards were purchased from ChromaDex (Irvine, CA). The solvents acetone, methanol, acetonitrile, trifluoroacetic acid, and acetic acid were HPLC grade (Fisher Scientific Co.). 2,2′-azobis(2-aminopropane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

2.2. Raspberry sample collection and preparation

Seven primocane fall-bearing raspberry cultivars were grown unsupported at the Kentland Virginia Tech Research farm, VA on irrigated plots using standard cultural and fertilization practices recommended for the region (Fernandez, Louws, Ballington, & Poling, 1998). The selected raspberry cultivars included both red and yellow fruit color: Nova (red), Dinkum (red), Heritage (red), Autumn Britten (red), Josephine, Anne (yellow), Fall Gold (yellow). Hand harvesting occurred during the month of September 2007 and only fully mature fruits were selected. Fruit samples were immediately transported at ambient temperatures to the Food Science and Technology facility at Virginia Tech (Blacksburg, VA). The samples were frozen upon arrival and subsequently lyophilized. The freeze-dried samples were ground and extracted with 50% acetone in a 1:10 (g/mL) ratio for 18 h with stirring. The acquired extracts were filtered through Whatman filter paper (No. 4). The filtrates were collected and further lyophilized for investigation.

2.3. Purification of the raspberry extracts

To examine the enzyme inhibition activities, it was necessary to remove the interfering sugars from the raspberry extracts. The purification was conducted on 6cc-Oasis® HLB cartridges (Waters, Milford, MA) using a solid phase extraction (SPE) procedure. In brief, a portion (150 mg) of the lyophilized crude extracts was reconstituted in the minimum volume of 20% methanol. The SPE cartridge was activated with methanol first and followed by 20% methanol. The extract was loaded onto the activated cartridge and sugars and other polar contaminants were removed by washing with 5% methanol. The phenolic compounds were eluted with absolute methanol and then lyophilized.

2.4. α-Amylase inhibition assay

The α-amylase inhibitory activity was determined using type VI-B porcine pancreatic α-amylase. A total of 500 μL of each raspberry extract and 500 μL of 0.02 mol/L sodium phosphate buffer (pH 6.9 with 6 mmol/L NaCl) containing α-amylase solution (0.5 mg/mL) was incubated at 25 °C for 10 min. After incubation, 500 μL of a 0.5% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 6 mmol/L NaCl) was added to each tube at timed intervals. The reaction was stopped by adding 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were then incubated 90 °C in a water bath for 10 min and cooled to room temperature. The reaction mixture was then diluted after the addition of 15 mL of distilled water, and absorbance was measured at 540 nm with a Victor^3 multilabel plate reader (Perkin–Elmer, Turku, Finland). The readings were compared with the controls, containing buffer in place of sample extract.

2.5. α-Glucosidase inhibition assay

The α-glucosidase activity was assayed using *p*-nitrophenyl-α-d-glucopyranoside (*p*NPG) as the substrate, which was hydrolyzed by α-glucosidase to release *p*-nitrophenol, a color agent that can be monitored at 405 nm (Babu et al., 2004). The assay was conducted by mixing 80 μL the sample solution (1 mg/mL) with 20 μL the enzyme solution (1 U/mL) and incubated at 37 °C for 3 min under shaking. After incubation, 100 μL of 4 mM *p*NPG solution in 0.1 M phosphate buffer (pH 6.8) was added and the reaction was performed at 37 °C. The release of *p*-nitrophenol from *p*NPG was monitored at 405 nm every minute for 75 min by a plate reader (Victor^3, Perkin–Elmer, Turku, Finland). The α-glucosidase activity was determined by measuring area under the curve (0–75 min) for each sample and compared with that of control.

2.6. Lipase inhibition assay

The inhibition of lipase activity was determined by measuring the amount of 4-metyhlumbelliferone product released by lipase using a Victor^3 multilabel plate reader (Perkin–Elmer, Turku, Finland) following a reported method with modification (Nakai et al., 2005). Pancreatic lipase (type II, from porcine pancreas) and 4-methylumbelliferyl oleate (4-MU oleate) served as the reac-

tion enzyme and substrate, respectively. The reaction mixture was prepared with 25 μL of the raspberry sample or lipase inhibitor (Orlistat, O4139, Sigma) and 25 μL of 16.7 U/mL lipase in Tris–HCl, pH 8.0 buffer solution. The reaction was initiated by adding 50 μL of 0.1 M 4-MU oleate in Tris–HCl, pH 8.0 buffer solution. After incubation at 37 °C for 30 min, the rate of release of the 4-methylumbeliferone product was measured with a plate reader (Perkin–Elmer, Turku, Finland) at an excitation wavelength of 355 nm and emission wavelength of 460 nm. The readings were compared with the controls, containing buffer in place of sample extract.

2.7. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was conducted to measure the peroxyl radical scavenging activity of each raspberry sample by using the water soluble vitamin E analog, Trolox, as the antioxidant standard according to the method reported previously (Zhou et al., 2007). In brief, a fluorescein stock solution (100 μmol/L) in phosphate buffer (75 mmol/L, pH 7.4) was prepared and kept at 4 °C in the dark. Fresh working fluorescein solution (100 mmol/L) was prepared daily by diluting the stock solution in phosphate buffer. Next, 200 μL of the working fluorescein solution were added to each 40μL of raspberry sample or Trolox standard prepared in phosphate buffer in a black 96-well plate and incubated for 20 min at 37 °C. The assay was initiated by adding the peroxyl radical generator prepared in phosphate buffer. Specifically, 35 μL of 2,2′-azobis-2-amidinopropane (AAPH, 0.36 mol/L) was added and the fluorescence was measured (λ_{ex} = 485 nm and λ_{em} = 535 nm) every minute using a Victor^3 multilabel plate reader (Perkin–Elmer, Turku, Finland) maintained at 37 °C until the reading had declined to less than 5% of the initial reading (e.g. 20 min). Standards and samples were run in triplicate. Results for ORAC were determined using a regression equation relating Trolox concentrations and the net area under the kinetic fluorescein decay curve. The ORAC value of each grape extract was expressed in μmol TE/g.

2.8. DPPH· scavenging assay

The DPPH· scavenging antioxidant capacity assay was determined by using a previously reported method (Zhou et al., 2007). The reaction mixture contained 100 μL of sample extract and 100 μL of 0.208 mmol/L DPPH· solution. The absorption at 515 nm was determined immediately after the reaction was initiated by gentle shaking. Each plate was read once every minute for 1.5 h. The relative DPPH· scavenging capacity for each raspberry cultivar sample was expressed as μmol TE/g.

2.9. Total phenolic content

The total phenolic content in the 50% acetone phenolic raspberry extract was determined by using a slightly modified method (Shetty, Curtis, Levin, Witkowsky, & Ang, 1995; Zhou, Su, & Yu, 2004). The lyophilized raspberry extracts were reconstituted in aqueous ethanol and mixed with Folin–Ciocalteu reagent. The absorbance was read at 725 nm using a spectrophotometer (Genesys UV–visible, Milton Roy, Inc., Rochester, NY). Appropriate concentrations of gallic acid solution were used to develop a standard curve. Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dried raspberry extract.

2.10. Total anthocyanin content

Each of the raspberry extracts was quantified for anthocyanin content using a pH differential method (Moyer, Hummer, Finn, Frei, & Wrolstad, 2002). In brief, 100 mL of 25 mM potassium chloride (pH 1.0) and 0.4 M sodium acetate (pH 4.5) buffer solutions were prepared. Each sample and standard (cyanidin 3-glucose) (Sigma, St. Louis, MA) was diluted first with 0.025 M potassium chloride buffer (pH 1.0) and the absorbance was measured at 520 nm and 700 nm against a reagent blank (distilled water). A second aliquot of each sample was diluted to the same value with 0.4 M sodium acetate buffer (pH 4.5) and measured at 520 and 700 nm. The total anthocyanin content was calculated and expressed in mg of cyanidin 3-glucoside equivalent per gram of dried raspberry 50% acetone extract.

2.11. HPLC analysis of phenolic acid composition

The phenolic acid profile for each raspberry cultivar was performed on a Waters 1525 HPLC system (Milford, MA) equipped with a photodiode array detector. Phenolic acids were separated on a Phenomenex Luna C18 column (250 mm × 4.6 mm, particle size 5 μm) (Phenomenex, Torrance, CA) using a linear gradient elution program with a mobile phase containing solvent A (acetic acid/H_2O, 2:98, v/v) and solvent B (acetic acid/acetonitrile/H_2O, 2:30:68, v/v/v) (Zhou, Laux, & Yu, 2004). The solvent gradient was linear, programmed from 10% to 100% with solvent B in 42 min with a flow rate of 1.0 mL/min. Identification of phenolic acids in each raspberry sample was accomplished by comparing the retention time and absorption spectra of peaks in raspberry samples to that of standard compounds. Quantification of individual phenolic acids was conducted using total area under each peak with external standards.

2.12. Fractionation and determination of active α-glucosidase inhibitors in 'Dinkum' raspberry extract

The 'Dinkum' raspberry extract was selected for this investigation because it showed the strongest α-glucosidase inhibitory activity among the tested raspberry extracts. The fractionation and identification of active α-glucosidase inhibitors in the extract was carried out using a bioactivity-driven approach (Fig. 1). The lyophilized extract was reconstituted in 10% methanol and fractionated into three major fractions, (1) mainly phenolic acids, (2) mainly anthocyanins and flavonols, and (3) mainly tannins, using Sephadex LH-20 open column chromatography. In brief, 10 g of Sephadex LH-20 (Sigma, St. Louis, MO) was hydrated for 4 h in 50 mL of water and then packed into a 60 mL glass column (Chemglass, Vineland, NJ) by elution with water. After balancing the column with 10% methanol, 4 mL of the constituted extract (25 mg/mL) was loaded to the column and eluted with 100 mL of 15% methanol/water (v/v) to collect phenolic acids, followed by 80% methanol/water (v/v) to elute the

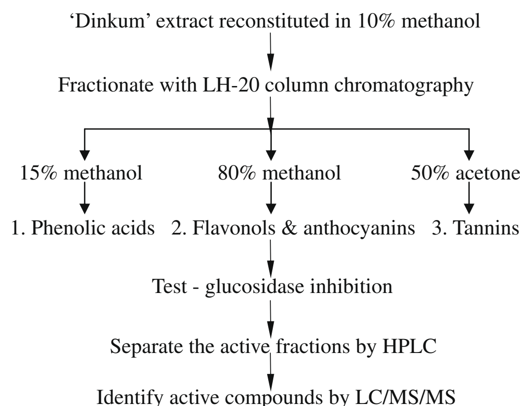


Fig. 1. Flow chart for the fractionation and determination of active α-glucosidase inhibitors in 'Dinkum' extract.

anthocyanins and flavonols, and finally by 50% acetone for elution of the tannins. The three fractions were collected separately and lyophilized. A part of the fractions was then reconstituted in dimethyl sulphoxide (DMSO) for determination of inhibitory activity on α-glucosidases. The active fractions (fractions 1 and 2) were further fractionated by HPLC and analysed by LC/MS/MS (liquid chromatograpy–tandem mass spectrometry). Specifically, the phenolic acid fraction was separated by the reverse phase HPLC using a C18 column (Porasil, 2.1 mm × 150 mm). The mobile phase consisted of 1% formic acid in water (solvent A) and acetonitrile (solvent B). A gradient program was applied as follows: 0–40 min: 0–30% solvent B; 40–65 min: 30–100% solvent B at a flow rate of 0.5 mL/min. The eluent was monitored at 280 nm. For the HPLC separation of anthocyanins and flavonols fraction, the binary mobile phases were 0.5% formic acid in water (solvent A) and 0.5% formic acid in acetonitrile (solvent B). The gradient was 10–85% B from 0 to 50 min. The detector was set to 520 nm for anthocyanins, and 370 nm for flavonols. The fractions after HPLC were collected based on the peaks and elution time intervals by a fraction collector (WFC II, Waters). All the fractions were dried in a Speedvac (Savant, Thermo Scientific, Waltham, MA) and reconstituted in DMSO for α-glucosidase test. The original fractions (fractions 1 and 2) and the HPLC fractions with the significant inhibitory activity were further analysed with LC/MS/MS. The chromatographic separation was performed using the same conditions developed in the HPLC separation. The HPLC column effluent was pumped directly without any split into a Finnigan LCQDUO mass spectrometer (Thermo Scientific, Waltham, MA) with electrospray ionization. The MS/MS parameters were as follows: positive mode; nebulizer, 45 psi; dry gas, 11.0 psi, dry temperature, 340 °C; MS/MS, scan from m/z 350 to 1500; ion trap, scan from m/z 100 to 1500; maximum accrual time, 100 ms.

2.13. Statistical analysis

All analyses were run at least in triplicate and were expressed as mean ± standard deviation (SD). Statistical analysis was performed with two-way analysis of variance using SPSS, version 10.0. The Fisher's least significant difference (LSD) at $P < 0.05$ was used to determine significant differences among samples. The relationship among samples' activities and their phenolic composition was conducted using Pearson's correlation coefficient ($P < 0.05$).

3. Results

3.1. Inhibitory effects on pancreatic lipase, α-amylase, and α-glucosidase

The phenol-rich extracts from the different cultivars of Virginia raspberries were tested for their ability to inhibit pancreatic α-amylase, α-glucosidase, and lipase. Under our experimental conditions, none of the seven purified raspberry extracts showed detectable inhibitory activity on pancreatic α-amylase or lipase (data not shown). However, all the extracts strongly inhibited activity of α-glucosidase (Fig. 2). Their inhibitory effects were further evaluated on different doses and the IC_{50}, the concentration that provides 50% inhibition, was obtained for each raspberry extract (Fig. 2). A low IC_{50} translates to a stronger α-glucosidase inhibition. There was a twofold difference on the IC_{50} values among cultivars (from 16.8 to 34.2 μg/mL). 'Dinkum' raspberry had the lowest IC_{50} of 16.8 μg/mL, followed by 'Autumn Britten' (17.2 μg/mL), 'Josephine' (18.0 μg/mL), 'Fall Gold' (20.3 μg/mL), and 'Anne' (23.8 μg/mL). The red colored 'Nova' and 'Heritage' fruit extracts showed the highest IC_{50} values of 33.9 and 34.2 μg/mL, respectively. The results suggested that the 'Dinkum' extract had the strongest inhibitory activity while the 'Heritage' extract was lowest against α-glucosidase. The potency of inhibition of α-glucosidase was not related to fruit color. For instance, both yellow cultivars, 'Anne' and 'Fall Gold', had similar inhibitory effects on α-glucosidase when compared to the red cultivars.

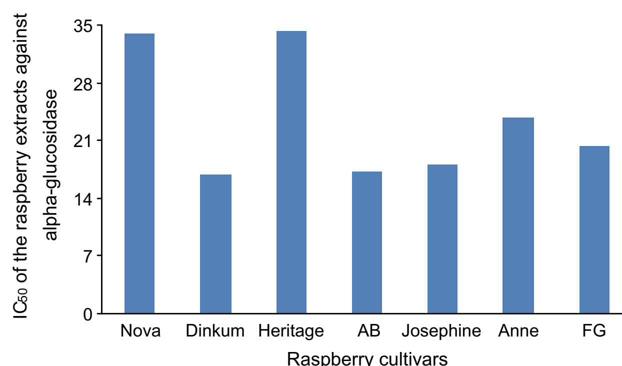


Fig. 2. IC_{50} of different raspberry cultivars on α-glucosidase. "AB" and "FG" represent 'Autumn Britten' and 'Fall Gold' raspberries, respectively. The IC_{50} is the concentration of the raspberry extracts that inhibit 50% of α-glucosidase activity.

3.2. ORAC

The ORAC assay measured the scavenging capacity of each raspberry cultivar against a peroxyl radical. The ORAC values ranged from 136.7 to 205.2 μmol TE/g dry weight (Fig. 3a). The variation of ORAC values is small although the significant difference was detectable among cultivars. The 'Autumn Britten' had the highest

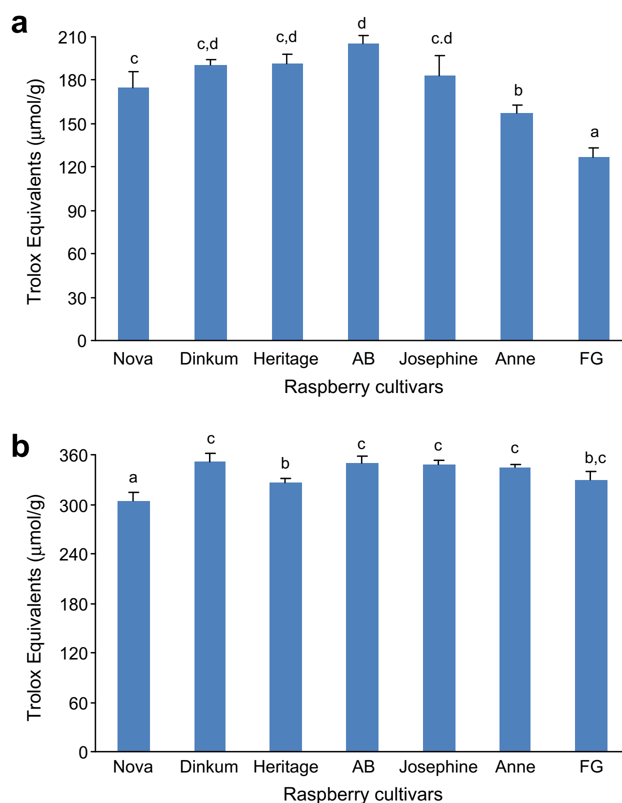


Fig. 3. ORAC (a) and DPPH radical scavenging activities (b) of different raspberry cultivars. "AB" and "FG" represent 'Autumn Britten' and 'Fall Gold' raspberries, respectively. ORAC values are expressed as μmol Trolox equivalents (TE)/g of dry weight of raspberries (mean ± SD, $n = 3$). The data marked by different letters are significantly different ($P < 0.05$).

ORAC value (205.2 µmol TE/g), followed 'Heritage' (191.3 µmol TE/g), 'Dinkum' (190.2 µmol TE/g), 'Josephine' (183.0 µmol TE/g), 'Anne' (177.1 µmol TE/g). 'Nova' and 'Fall Gold' had the lowest ORAC values of 157.2 and 136.7 µmol of TE/g, respectively.

3.3. DPPH· scavenging capacity

DPPH· assay was used as the second measure to evaluate antioxidant activities of the raspberry extracts. Under experimental conditions, the DPPH· scavenging capacity of the raspberry cultivars ranged from 305 to 351 µmol TE/g dry weight (Fig. 3b). The 'Dinkum' extract had the highest activity against DPPH· (351.3 µmol TE/g), followed by 'Autumn Britten', 'Josephine', 'Anne', 'Fall Gold', 'Heritage' (349.6, 348.9, 344.7, 330.4, and 326.0 µmol TE/g, respectively), and finally 'Nova' with a significant lower value of 304.5 µmol TE/g.

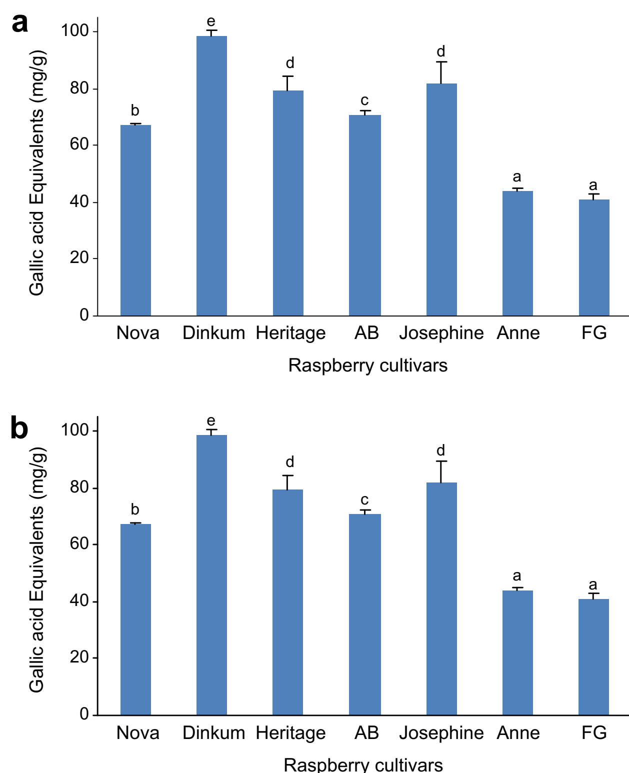


Fig. 4. Total phenolic (a) and anthocyanin (b) content of different raspberry cultivars. "AB" and "FG" represent 'Autumn Britten' and 'Fall Gold' raspberries, respectively. Results are expressed as mg gallic acid equivalents per gram of dry weight of raspberries (mean ± SD, $n = 3$). The data marked by different letters are significantly different ($P < 0.05$).

3.4. Total phenolic content

The TPC of the seven raspberry cultivars varied significantly from 40.9 to 98.5 mg of gallic acid equivalents (GAE)/g dry weight (Fig. 4a). The 'Dinkum' extract had the highest total phenolic content (98.5 mg GAE/g dry weight) of the measured raspberries followed by the other red colored cultivars including: Josephine (81.7 mg GAE/g), 'Heritage' (79.2 mg GAE/g), 'Autumn Britten' (70.7 mg of GAE/g), and then 'Nova' (66.9 mg of GAE/g). Similar to a previous report, 'Anne' and 'Fall Gold' (yellow cultivars) had the lowest TPC (44.1 and 40.9 mg of GAE/g, respectively) (Liu et al., 2002a).

3.5. Total anthocyanin content

The total anthocyanin content of the seven different cultivars of raspberries is expressed as mg cyanidin 3-glucoside equivalents (CGE)/g dry weight and their values ranged from 0.1–9.5 CGE/g (Fig. 4b). The red Nova raspberry had the highest total anthocyanin content (9.5 CGE/g) followed by the other red colored cultivars including 'Josphine' (9.4 mg CGE/g), 'Dinkum' (8.6 mg CGE/g), 'Heritage' (7.9 mg CGE/g), and 'Autumn Britten' (7.0 mg CGE/g). The yellow colored raspberries ('Anne' and 'Fall Gold') had lower total anthocyanin contents of 0.2 and 0.1 mg CGE/g, respectively, as expected. These findings are in agreement with the well establish literature in that the anthocyanins play a key role in the determination of the color of red raspberry fruits.

3.6. HPLC analysis of phenolic acid composition

Free phenolic acids in the raspberries were extracted with 50% acetone and further purified by SPE prior to HPLC analysis. The total amounts of free phenolic acids varied from 157.3 to 713.5 µg/g dry weight among different raspberry cultivars (Table 1). 'Anne' and 'Dinkum' contained exceptionally higher phenolic acids compared to other raspberries, while 'Fall Gold' and 'Autumn Britten' had the lowest phenolic acid content. The phenolic acid profiles of selected raspberries were also remarkably different. However, in general, ferulic and gentisic acids were the major phenolic acids in the raspberries. Other phenolic acids detected in the raspberry samples include gallic, chlorogenic, ellagic, *p*-hydroxy benzoic, vanillic, caffeic, and *p*-coumaric acids.

3.7. Fractionation and determination of active α-glucosidase inhibitors in 'Dinkum' extract

The initial fractionation with LH-20 column chromatography afforded three major fractions. The fraction 1 (phenolic acids) and fraction 2 (anthocyanins and flavonols) showed strong α-glucosidase inhibition (IC_{50} 25.4 µg/mL and 11.6 µg/mL, respectively) and were identified as the active fractions. However, the fraction 3 (tannins) showed only minimum inhibition on α-glucosidase

Table 1
Free phenolic acid composition of different raspberry cultivars determined by HPLC.

Phenolic acids	Dinkum (µg/g)	Nova (µg/g)	Heritage (µg/g)	Autumn Britten (µg/g)	Josephine (µg/g)	Anne (µg/g)	Fall Gold (µg/g)
Gallic acid	ND*	5.0 ± 0.1	1.2 ± 0.2	6.1 ± 0.4	7.2 ± 1.0	18.2 ± 0.3	2.7 ± 0.4
Chlorogenic acid	ND	1.4 ± 0.4	0.5 ± 0.1	2.0 ± 0.1	2.9 ± 0.3	1.1 ± 0.1	ND
Gentisic acid	150.9 ± 5.6	263.8 ± 4.4	23.0 ± 1.8	139.0 ± 5.6	124.5 ± 27.1	551.6 ± 21.7	116.6 ± 17.5
Ellagic acid	6.2 ± 3.2	0.4 ± 0.1	ND	12.0 ± 2.5	5.8 ± 1.2	21.3 ± 2.4	18.6 ± 3.1
4-OH benzoic acid	38.7 ± 1.4	17.9 ± 2.8	14.3 ± 1.1	11.1 ± 1.1	17.3 ± 2.4	115.6 ± 5.4	2.1 ± 0.3
Vanillic acid	19.2 ± 2.7	ND	2.2 ± 0.4	5.3 ± 0.2	7.5 ± 1.4	1.6 ± 0.3	4.6 ± 0.2
Caffeic acid	46.1 ± 1.9	17.1 ± 0.8	10.6 ± 0.7	10.6 ± 0.9	112.4 ± 10.7	44.9 ± 3.4	9.3 ± 1.5
p-Coumaric acid	17.6 ± 1.8	6.2 ± 0.1	2.9 ± 0.7	1.4 ± 0.1	3.2 ± 0.7	23.9 ± 2.5	1.7 ± 0.2
Ferulic acid	379.4 ± 9.6	7.5 ± 0.7	238.7 ± 3.9	31.4 ± 1.4	165.5 ± 8.8	20.8 ± 7.1	1.9 ± 0.1
Total detected phenolic acids	658.2	386.5	293.3	289.0	346.3	713.5	157.3

ND*: not detectable under the experimental conditions.

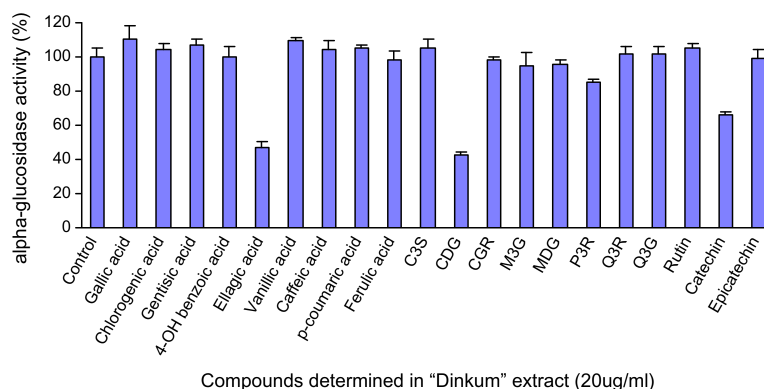


Fig. 5. Inhibitory activities of the components determined 'Dinkum' against α-glucosidase. C3S, CDG, CGR, M3G, MDG, P3R, Q3R, and Q3G represent cyanidin-3-sophoroside, cyanidin-diglucoside, cyanidin-glucosylrutinoside, malvidin-glucoside, malvidin-diglucoside, pelargonidin-3-rutinoside, quercetin-3-rutinoside, and quercetin-3-glucoside. The enzyme assays were conducted under the same conditions for all the components (20 μg/mL).

(IC_{50} > 300 μg/mL). The fractions 1 and 2 were further separated with HPLC and analysed with LC/MS/MS to identify active components (Fig. 5). In fractions 1, seven phenolic acids were determined and ellagic acid was identified as the active α-glucosidase inhibitor with an IC_{50} of 18.4 μg/mL. In fraction 2, six anthocyanins (cyanidin-3-sophoroside, cyanidin-diglucoside, cyanidin-glucosylrutinoside, malvidin-glucoside, malvidin-diglucoside, and pelargonidin-3-rutinoside), and other flavonoids including quercetin-3-rutinoside, quercetin-3-glucoside, epicatechin, catechin, rutin were determined. Among these compounds, cyanidin-diglucoside, pelargonidin-3-rutinoside, and catechin were identified as the active α-glucosidase inhibitors with IC_{50} of 14.7, 64.5, and 39.2 μg/mL, respectively.

4. Discussion

Phenolic compounds in berries have been well characterised as natural antioxidants, which are believed to play a major role in certain health benefits attributed to a diet rich in those fruits. These naturally occurring phenolic antioxidants encompass a diverse range of chemical classes that protect against the damage caused by reactive oxygen species to DNA, membrane, and cellular components (Shahidi, 1997). In addition to antioxidant protection, berry polyphenols are also reported to exert multiple biological activities. For example, evidence suggests that polyphenols from berries are involved in the inhibitory activities of metabolizing enzymes (McDougall & Stewart, 2005). Recently, a raspberry extract was found to be an effective inhibitor of starch digesting enzymes α-glucosidase and α-amylase (McDougall et al., 2005), suggesting that specific raspberry cultivars may help reduce starch digestion and thus provide a beneficial effect on diabetes by ameliorating postprandial glycemic response. This effect along with the known antioxidant activity found in raspberries may exert dual benefits for preventing and treating diabetes, a disease affecting millions people worldwide.

The objective of this study was to examine different raspberry cultivars for their inhibitory effect on digestive enzymes and antioxidant properties and to potentially identify specific cultivars that may be particularly useful functional food candidates for improving blood glucose control. Seven raspberry cultivars in this study were assessed for their inhibition of three digestive enzymes: pancreatic α-amylase, α-glucosidase, and lipase. Although there are no reports available on effects of raspberry on lipase activity, lipase was included in our study for comparison with other digestive enzymes and also because previous research suggested that fruit proanthocyanidins may inhibit gastrointestinal lipase activity (Moreno et al., 2003), a therapeutic target for weight control through reduced dietary digestion and absorption (Hill et al., 1999). The selected raspberry cultivars showed no detectable inhibitory effect on pancreatic lipase and α-amylase activities. This was inconsistent with the previous report that that the extract of Glen Ample raspberry inhibited activity of pancreatic α-amylase (McDougall et al., 2005). The sample preparation procedures were similar in both studies. Therefore, we suspect that the inconsistency may be attributed to the cultivar differences. It is known that α-amylase and α-glucosidase, both under glycoside hydrolase family 13, share a common reaction mechanism and several short conserved sequence (Inohara-Ochiai et al., 1997). Interestingly, all the raspberry extracts potently inhibit activities of α-glucosidase with IC_{50} ranging from 16.8–34.3 μg/mL but showed no inhibition on α-amylase, suggesting the raspberry extracts may specifically target α-glucosidase. In this study the 'Dinkum' fruit extract had the strongest inhibitory activity, which was more than 2 times better than that of the 'Nova' and 'Heritage' extracts, indicating significant cultivar differences. Further HPLC and LC/MS/MS analysis on the 'Dinkum' extract led to the identification of four active α-glucosidase inhibitors: ellagic acid, cyanidin-diglucoside, pelargonidin-3-rutinoside, and catechin. It should be noted that a number of other unknown components (peaks separated by HPLC) also showed strong inhibition on α-glucosidase, which remained to be elucidated by further NMR (nuclear magnetic resonance) experiments. Our results also suggest that the specific inhibitory mechanism of the raspberry extracts on α-glucosidase differs from that of the commercially available oral hypoglycyemic agent, acarbose, which inhibits both α-amylase and α-glucosidase (Kim et al., 1999). Collectively, this data suggest that the raspberry extracts can specifically inhibit α-glucosidase activity and therefore, may provide an alternative nutritional option for type 2 diabetes prevention and treatment.

Antioxidant properties of raspberries were evaluated by ORAC and $DPPH^{\cdot}$ scavenging activities. The ORAC values of the raspberry extracts significantly ranged from 136.7 to 205.2 μmol TE/g dry weight fruit, suggesting all raspberry samples have remarkable antioxidant activity against peroxyl radicals. These ORAC values were higher than that of 'Autumn Bliss', another fall-bearing primocane cultivar cultivated in Maryland (106.3 μmol TE /g dry mass) (Wang & Lin, 2000). Other *Rubus* species of raspberries such as *R. innominatus* and *R. niveus* were also reported to have ORAC values in the range of 13 to 45 μmol TE/g fresh weight (Hosseinian et al., 2007; Moyer et al., 2002), which were comparable to our results when taking into consideration that raspberries are comprised of 85%-90% water. The $DPPH^{\cdot}$ scavenging activities of the raspberry extracts ranged from 304.5 to 351.3 μmol TE/g dry weight. There was no significant correction between ORAC and

DPPH· scavenging activities of the raspberry cultivars in this study. This may be because ORAC and DPPH· scavenging assays are involved in different reaction mechanisms; the former was based on hydrogen atom transfer reactions, while the later was based on an electron transfer mechanism (Ou, Hampsch-Woodill, & Prior, 2001). Thus, inclusion of different assays is important to make precise estimates and comparisons of antioxidant activities from different samples. Collectively, this study adds to the body of evidence that a wide variety of cultivated raspberries are good sources of natural antioxidants with remarkable radical scavenging capacity. However, we found no significant correlation ($P < 0.05$) between the antioxidant activities (both ORAC and DPPH· scavenging activity) of the tested raspberry cultivars and their α-glucosidase inhibitory activities.

The total phenolic content, total anthocyanins, and phenolic acids in the raspberries were also determined. The selected raspberries had TPC values between 40.9 and 98.5 mg GAE/g dry weight, which were comparable to the previous report for 'Heritage' and 'Anne' cultivars (Liu et al., 2002a) and higher than that of 'Autumn Bliss' (14.1 mg GAE/g) (Wang & Lin, 2000). The 'Dinkum' raspberry had the highest TPC. Although 'Dinkum' also possessed the strongest inhibitory activity against α-glucosidase, the Pearson's correlation test suggested no significant correlation between the TPC and α-glucosidase inhibition among raspberry extracts. The total anthocyanin content in the raspberries varied dramatically, ranging from 0.1 to 9.5 CGE/g dry weight with dark-red raspberries having the highest anthocyanin content. This was in agreement with a previous report that also found that anthocyanins were a major contributor to raspberry color. The dark-red 'Heritage' fruit had a much higher anthocyanin content than yellow 'Anne' cultivar (Liu et al., 2002a). This finding was expected considering that anthocyanin is a major pigment responsible for dark/red or purple coloration in plant tissues. However, in contrast to the same study, we found that the TPC of raspberries was not correlated to their anthocyanin content. Plant polyphenolic extracts have been reported to inhibit α-glucosidase activity, especially in those anthocyanins-rich extracts of berries and blackcurrant (McDougall et al., 2005; McDougall & Stewart, 2005; Yuste et al., 1992). Individual anthocyanin compounds were shown to be effective inhibitors of α-glucosidase and exhibit an anti-hyperglycemic effect in rats (Matsui et al., 2002). In this study, two anthocyanins, cyanidin-diglucoside and pelargonidin-3-rutinoside in 'Dinkum' were identified as the inhibitors of α-glucosidase. However, the anthocyanin content in the raspberries was not correlated with their enzyme inhibitory activity. Moreover, 'Anne' and 'Fall Gold' raspberries which contained marginal anthocyanins showed potent inhibition on α-glucosidase. We further determined phenolic acids, another major group of phenolic compounds in the raspberries. Nine phenolic acids were quantified in the selected raspberries. From the profile of phenolic acids that differences exists in terms of bioactive constituents among raspberry cultivars.

In summary, this study demonstrated that the different fall-bearing primocane raspberries possessed distinct antioxidant capacity and potent α-glucosidase inhibitory activity. The red cultivars 'Dinkum' and 'Josephine' cultivars had consistently higher ORAC, DPPH, total phenolic content, and enzyme inhibitory effects as compared to the other five cultivars, suggesting specific raspberries may provide a protective effect for diabetes and relevant complications through dual protection of antioxidant and inhibition of starch digestion. The inhibition of α-glucosidase by some raspberry extracts appears to be specific as they did not inhibit other digestive enzymes such as pancreatic α-amylase and lipase. Four phenolic compounds, ellagic acid, cyanidin-diglucoside, pelargonidin-3-rutinoside, and catechin, were identified as the active α-glucosidase inhibitory components in raspberries. Further investigation is warranted to explore their potential therapeutic effects on post-meal glycemic response by limiting starch digestion.

References

Babu, K., Tiwari, A. K., Srinivas, P. V., Ali, A. Z., Raju, B., & Rao, J. M. (2004). Yeast and mammalian [α]-glucosidase inhibitory constituents from Himalayan rhubarb *Rheum emodi* Wall. ex Meisson. *Bioorganic and Medicinal Chemistry Letters, 14*(14), 3841–3845.

Beekwilder, J., Hall, R. D., & de Vos, C. H. (2005). Identification and dietary relevance of antioxidants from raspberry. *Biofactors, 23*(4), 197–205.

Beekwilder, J., Jonker, H., Meesters, P., Hall, R. D., vanderMeer, I. M., & RicdeVos, C. H. (2005). Antioxidants in raspberry: On-line analysis links antioxidant activity to a diversity of individual metabolites. *Journal of Agricultural and Food Chemistry, 53*(9), 3313–3320.

Chiasson, J. L., Josse, R. G., Gomis, R., Hanefeld, M., Karasik, A., & Laakso, M. (2002). Acarbose for prevention of type 2 diabetes mellitus: The STOP-NIDDM randomised trial. *Lancet, 359*(9323), 2072–2077.

Estaquio, C., Castetbon, K., Kesse-Guyot, E., Bertrais, S., Deschamps, V., Dauchet, L., et al. (2008). The French National Nutrition and Health Program score is associated with nutritional status and risk of major chronic diseases. *Journal of Nutrition, 138*(5), 946–953.

Fernandez, G. E., Louws, F. J., Ballington, J. R., & Poling, E. B. (1998). Growing raspberries in North Carolina. *North Carolina State University extension publication AG-569*.

Hill, J. O., Hauptman, J., Anderson, J. W., Fujioka, K., O'Neil, P. M., Smith, D. K., et al. (1999). Orlistat, a lipase inhibitor, for weight maintenance after conventional dieting: A 1-y study. *American Journal of Clinical Nutrition, 69*(6), 1108–1116.

Hosseinian, F. S., Li, W., Hydamaka, A. W., Tsopmo, A., Lowry, L., Friel, J., et al. (2007). Proanthocyanidin profile and ORAC values of Manitoba berries, chokecherries, and seabuckthorn. *Journal of Agricultural and Food Chemistry, 55*(17), 6970–6976.

Inohara-Ochiai, M., Nakayama, T., Goto, R., Nakao, M., Ueda, T., & Shibano, Y. (1997). Altering substrate specificity of *Bacillus* sp. SAM1606 α-glucosidase by comparative site-specific mutagenesis. *Journal of Biological Chemistry, 272*(3), 1601–1607.

Kim, M.-J., Lee, S.-B., Lee, H.-S., Lee, S.-Y., Baek, J.-S., Kim, D., et al. (1999). Comparative study of the inhibition of [α]-glucosidase, [α]-amylase, and cyclomaltodextrin glucanosyltransferase by acarbose, isoacarbose, and acarviosine–glucose. *Archives of Biochemistry and Biophysics, 371*(2), 277–283.

Liu, M., Li, X. Q., Weber, C., Lee, C. Y., Brown, J., & Liu, R. H. (2002a). Antioxidant and antiproliferative activities of raspberries. *Journal of Agricultural and Food Chemistry, 50*(10), 2926–2930.

Liu, M., Li, X. Q., Weber, C., Lee, C. Y., Brown, J., & Liu, R. H. (2002b). Antioxidant and antiproliferative activities of raspberries. *Journal of Agricultural and Food Chemistry, 50*(10), 2926–2930.

Matsui, T., Ebuchi, S., Fukui, K., Matsugano, K., Terahara, N., & Matsumoto, K. (2004). Caffeoylsophorose, a new natural α-glucosidase inhibitor, from red vinegar by fermented purple-fleshed sweet potato. *Bioscience, Biotechnology, and Biochemistry, 68*(11), 2239–2246.

Matsui, T., Ebuchi, S., Kobayashi, M., Fukui, K., Sugita, K., Terahara, N., et al. (2002). Anti-hyperglycemic effect of diacylated anthocyanin derived from *Ipomoea batatas* cultivar ayamurasaki can be achieved through the alpha-glucosidase inhibitory action. *Journal of Agricultural and Food Chemistry, 50*(25), 7244–7248.

Matsui, T., Ueda, T., Oki, T., Sugita, K., Terahara, N., & Matsumoto, K. (2001). α-Glucosidase inhibitory action of natural acylated anthocyanins. 2. α-Glucosidase inhibition by isolated acylated anthocyanins. *Journal of Agricultural and Food Chemistry, 49*(4), 1952–1956.

McDougall, G. J., Ross, H. A., Ikeji, M., & Stewart, D. (2008). Berry extracts exert different antiproliferative effects against cervical and colon cancer cells grown in vitro. *Journal of Agricultural and Food Chemistry, 56*(9), 3016–3023.

McDougall, G. J., Shpiro, F., Dobson, P., Smith, P., Blake, A., & Stewart, D. (2005). Different polyphenolic components of soft fruits inhibit α-amylase and α-glucosidase. *Journal of Agricultural and Food Chemistry, 53*(7), 2760–2766.

McDougall, G. J., & Stewart, D. (2005). The inhibitory effects of berry polyphenols on digestive enzymes. *Biofactors, 23*(4), 189–195.

Moreno, D. A., Ilic, N., Poulev, A., Brasaemle, D. L., Fried, S. K., & Raskin, I. (2003). Inhibitory effects of grape seed extract on lipases. *Nutrition, 19*(10), 876–879.

Moyer, R. A., Hummer, K. E., Finn, C. E., Frei, B., & Wrolstad, R. E. (2002). Anthocyanins, phenolics, and antioxidant capacity in diverse small fruits: Vaccinium, rubus, and ribes. *Journal of Agricultural and Food Chemistry, 50*(3), 519–525.

Mullen, W., Stewart, A. J., Lean, M. E., Gardner, P., Duthie, G. G., & Crozier, A. (2002). Effect of freezing and storage on the phenolics, ellagitannins, flavonoids, and antioxidant capacity of red raspberries. *Journal of Agricultural and Food Chemistry, 50*(18), 5197–5201.

Naczk, M., & Shahidi, F. (2006). Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis, 41*(5), 1523–1542.

Nakai, M., Fukui, Y., Asami, S., Toyoda-Ono, Y., Iwashita, T., Shibata, H., et al. (2005). Inhibitory effects of oolong tea polyphenols on pancreatic lipase in vitro. *Journal of Agricultural and Food Chemistry, 53*(11), 4593–4598.

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

Seeram, N. P., Adams, L. S., Zhang, Y., Lee, R., Sand, D., Scheuller, H. S., et al. (2006). Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *Journal of Agricultural and Food Chemistry, 54*(25), 9329–9339.

Shahidi, F. (1997). *Natural antioxidants: Chemistry, health effects and applications*. Champaign, IL: AOCS Press. pp. 97–149.

Shahidi, F., & Wanasundara, P. K. (1992). Phenolic antioxidants. *Critical Reviews in Food Science and Nutrition, 32*(1), 67–103.

Shetty, K., Curtis, O. F., Levin, R. E., Witkowsky, R., & Ang, W. (1995). Prevention of verification associated with in vitro shoot culture of oregano (*Origanum vulgare*) by *Pseudomonas* spp. *Journal of Plant Physiology, 147*, 447–451.

Stevenson, D. E., & Hurst, R. D. (2007). Polyphenolic phytochemicals – just antioxidants or much more? *Cellular and Molecular Life Sciences, 64*(22), 2900–2916.

Toeller, M. (1994). α-Glucosidase inhibitors in diabetes: Efficacy in NIDDM subjects. *European Journal of Clinical Investigation, 24*(Suppl 3), 31–35.

van de Laar, F. A., Lucassen, P. L., Akkermans, R. P., van de Lisdonk, E. H., Rutten, G. E., & van Weel, C. (2005). α-Glucosidase inhibitors for patients with type 2 diabetes: Results from a Cochrane systematic review and meta-analysis. *Diabetes Care, 28*(1), 154–163.

Villegas, R., Salim, A., Flynn, A., & Perry, I. J. (2004). Prudent diet and the risk of insulin resistance. *Nutrition, Metabolism and Cardiovascular Diseases, 14*(6), 334–343.

Wang, S. Y., & Lin, H. S. (2000). Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *Journal of Agricultural and Food Chemistry, 48*(2), 140–146.

Weisburger, J. H. (2002). Lifestyle, health and disease prevention: The underlying mechanisms. *European Journal of Cancer Prevention, 11*(Suppl. 2), S1–S7.

Wolfe, K. L., Kang, X., He, X., Dong, M., Zhang, Q., & Liu, R. H. (2008). Cellular antioxidant activity of common fruits. *Journal of Agricultural and Food Chemistry, 56*(18), 8418–8426.

Yuste, P., Longstaff, M., & McCorquodale, C. (1992). The effect of proanthocyanidin-rich hulls and proanthocyanidin extracts from bean (*Vicia faba* L.) hulls on nutrient digestibility and digestive enzyme activities in young chicks. *British Journal of Nutrition, 67*(1), 57–65.

Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., Vinson, J. A., & Bagchi, D. (2007). Berry anthocyanins as novel antioxidants in human health and disease prevention. *Molecular Nutrition and Food Research, 51*(6), 675–683.

Zhou, K., Hao, J., Griffey, C., Chung, H., O'Keefe, S. F., Chen, J., et al. (2007). Antioxidant properties of Fusarium head blight-resistant and -susceptible soft red winter wheat grains grown in Virginia. *Journal of Agricultural and Food Chemistry, 55*(9), 3729–3736.

Zhou, K., Laux, J. J., & Yu, L. (2004). Comparison of Swiss red wheat grain and fractions for their antioxidant properties. *Journal of Agricultural and Food Chemistry, 52*(5), 1118–1123.

Zhou, K., Su, L., & Yu, L. L. (2004). Phytochemicals and antioxidant properties in wheat bran. *Journal of Agricultural and Food Chemistry, 52*(20), 6108–6114.