Comparison of α -amylase, α -glucosidase and lipase inhibitory activity of the phenolic substances in two black legumes of different genera

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ABSTRACT

Antioxidant-rich plant foods can inhibit starch and lipid digestions that are relevant to diabetes management. Two high-antioxidant black legumes, black soybean (*Glycine max*) and black turtle bean (*Phaseolus vulgaris*), belonging to two different genera were used to investigate their capacity against digestive enzymes. Phenolic substances were compared in crude, semi-purified extracts (semi-purified by XAD-7 column), and fractions (fractionated by Sephadex LH-20 column) from these two legumes. In addition, their antioxidant capacities and abilities to inhibit digestive enzymes were characterized. Results showed that Fraction V from black soybean was the most effective (IC₅₀: 0.25 mg/ml) against α -amylase; Fraction V from black turtle bean was the most potent (IC₅₀: 0.25 μ g/ml) against α -glucosidase; Fraction IV from black turtle bean was the most powerful (IC₅₀: 76 μ g/ml) against lipase. Of the pure phenolic compounds tested, myricetin showed the highest inhibition of α -amylase, α -glucosidase and lipase (IC₅₀: 0.38 mg/ml, 0.87 μ g/ml and 15 μ g/ml, respectively).

Keywords: Phenolic compounds, lipase, α -glucosidase and α -amylase inhibition

Research Highlights

1. Polyphenols in two legumes were fractionated by two-step chromatography.
2. Three digestive enzymes were inhibited by legume extracts and fractions.
3. Of the compounds tested, myricetin showed the lowest IC₅₀ values against three digestive enzymes.

1. Introduction

Diabetes affects 415 million people worldwide and this number is projected to rise to 642 million by 2040 (IDF Diabetes Atlas, 7th edition). Diabetes is partly caused by the excessive presence of carbohydrates in the diet. Starch digestion in mammals is mainly carried out by α -amylase and α -glucosidase (Rossi et al., 2006). Inhibition of starch digestive enzymes or glucose transporters can suppress postprandial hyperglycemia by reducing the rate of glucose release and absorption in the small intestine (Hanhineva et al., 2010). In addition, a fat-enriched diet and sedentary lifestyle also contributes to the prevalence of type-II diabetes (Cani et al., 2008). Epidemiological studies indicate that the consumption of phenolic-rich foods is inversely correlated with the prevalence of cardiovascular diseases (Corder et al., 2006) and type-II diabetes (Nwosu, Morris, Lund, Stewart, Ross, & McDougall, 2011). Growing evidence indicates that polyphenols contained in berries, vegetables, nuts and tea possess many health-promoting and disease-preventing properties (Roopchand, Kuhn, Rojo, Lila, & Raskin, 2013). Phenolics can inactivate α -amylase, α -glucosidase and lipase through non-specific binding to enzymes (Zhang et al., 2015). Most reported studies used crude phenolic extracts, in which the major contents of crude phenolic extracts are sugars. So far, none of the reported studies have used purified or semi-purified phenolic **extractions** isolated from common foods. Therefore, analyzing the **enzyme-inhibition abilities of** the semi-purified and purified phenolic **fractions** would aid understanding of **the stucture-activity relationship**.

Black turtle bean (*Phaseolus vulgaris* L.) and black soybean (*Glycine max* L.) belong to two distinctly different scientific genera. Both of them are very popular in the Oriental diet. However, consumers are often confused between the two because of their similar appearance. To date, there have been no systematic studies to compare chemical composition differences in their phenolic substances with respect to their

ability to inhibit digestive enzymes. Therefore, our objective was to compare three phenolic substances (total phenolic content, total flavonoids, and condensed tannin) in crude, semi-purified extracts (by XAD-7 column chromatography) and fractions (separated the phenolic substances in the semi-purified extracts by a Sephadex LH-20 column) isolated from black turtle bean and black soybean, and to compare their antioxidant capacities and their abilities to inhibit α -amylase, α -glucosidase and lipase *in vitro*. Such information is useful for the consumer in order to make choices between black legumes based on health improving properties.

2. Materials and methods

2.1 Materials

Black turtle bean and black soybean were obtained from Goya (Secaucus, NJ, US) and all materials were stored at -20°C until use.

2.2 Chemicals

Sixteen HPLC grade phenolic acids: gallic, protocatechuic, 2,3,4-trihydroxybenzoic, p-hydroxybenzoic, gentistic, vanillic, caffeic, chlorogenic, syringic, p-coumaric, m-coumaric, o-coumaric, ferullic, salicylic, sinapic, and trans-cinnamic acid; 3 aldehydes: vanillin, syringaldehyde, and protocatechualdehyde; seven flavan-3-ols: (+)-catechin, catechin gallate, gallic catechin gallate, (+)-epicatechin, epigallocatechin, epicatechingallate, and epigallocatechin gallate; and 12 flavonols or flavones: quercetin, quercetin-3-O-glucoside, quercetin-3-rutinoside, kaempferol, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside, myricetin, luteolin, apigenin, resveratrol, polydatin and rutin were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A) as phenolic acid and flavonoid standards. HPLC grade trifluoroacetic acid (TFA) was obtained from Sigma Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A). Lipase from porcine pancreas (EC

3.1.1.3), α -amylase from porcine pancreas (EC 3.2.1.1), α -glucosidase from *saccharomyces cerevisiae* (EC 3.2.1.20), Type-1 α -amylase inhibitor from *Triticum aestivum* (protein mixture, Sigma product No. A1520), lipase inhibitor URB602 (mw: 295.38, Sigma product No. U3010), α -glucosidase inhibitor voglibose (clinical used drug, mw: 267.28), and other chemicals of analytical grade were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A).

2.3. Crude phenolic extraction

The extraction method was in accordance with our previous study (Xu & Chang, 2007). One gram of bean powder was extracted with a 10 ml solution of acetone/acetic acid/ water (70/0.5/29.5, v/v/v) and shaken at 150 rpm in a VWR standard analog shaker (West Chester, PA, U.S.A.) at room temperature for 3 h. The mixture was centrifuged using a Thermo Legend X1R centrifuge (Thermo Scientific Inc. Waltham, MA, U.S.A.) at $12000 \times g$ for 15 min. The residue was re-extracted for 12 h with 10 ml extraction solution. After centrifugation, the two supernatants were combined and concentrated using a rotary evaporator (BÜCHI Labortechnik AG, Switzerland) under vacuum at 38 °C to remove organic solvent. After freeze-drying, the extract powder was stored at -20 °C until use.

2.4. Purification of crude extraction

Sugar removal was performed by column chromatography using Amberlite® XAD-7 resin as the packing material, as in our earlier study (Zou, Chang, Gu, & Qian, 2011) with some modifications. Four grams of crude extract were dissolved in 20 ml of distilled water by vortexing vigorously and centrifuged to remove the insoluble components. The residue was re-dissolved in 5 ml distilled water, and centrifuged. The supernatants were combined and filtered through a 2 μ m membrane to obtain a clear solution. The clear solution was gently poured into the column (40 $\times$ 2.6 cm, i.d.,

bed volume (BV) = 180 ml) and eluted with distilled water at a speed of 1.5 BV/h. A flow chart for fractionation of black soybean and black turtle bean phenolics is shown in Figure 1. The resin was washed with 2 BV of distilled water, and then 80% methanol was used to elute the phenolic compounds at a speed of 3 BV/h. Methanol in the effluent was removed using a rotary evaporator under vacuum at 38 °C; and the concentrated extract was freeze-dried. The powder was designated as semi-purified extract, and stored at -20 °C until use.

Column fractionation of semi-purified extract was carried out using our previously published method with minor modifications (Zou, Chang, Gu, & Qian, 2011). Elution curves of fractionation of semi-purified black soybean and black turtle bean are shown in the supplementary material (Figure 1). One-hundred milligrams of semi-purified extract were dissolved in 1 ml distilled water and vortexed vigorously. The dispersion was filtered through a 2 µm membrane to remove insoluble residue. The filtrate was loaded onto a column (100 × 1.6 cm, i.d., BV= 200 ml) packed with Sephadex LH-20. The column was eluted with distilled water, 50% ethanol, and 50% acetone sequentially at a flow rate of 0.8 ml/min. The elution was monitored at 280 nm by an UV detector, which was connected to the Sephadex LH-20 column. The elution protocols were different between these two legumes, because black turtle bean had a higher condensed tannin content than black soybean, which meant that the last fraction of black turtle bean was more non-polar than that of black soybean. Therefore, the last fraction of black turtle bean needed to be eluted by a more non-polar solvent.

2.5. Total phenolic content determination

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method and following the procedure by Xu and Chang (2007) except that the volumes of solutions were decreased proportionally. Two-hundred microliters of the reaction

mixture were added into a 96-well plate, and the absorbance was measured with a plate reader (Molecular Devices, CA, U.S.A.) at 765 nm. Total phenolic content was expressed as gallic acid equivalents per gram of dry extracts or fractions (mg of GAE/g).

2.6. Total flavonoid content determination

Total flavonoid content was determined according to a previous method reported by our laboratory (Xu & Chang, 2007) except that the volumes of solutions were decreased proportionally. Two-hundred microliters of the mixture were added into a 96-well plate, and the absorbance was measured with a plate reader at 510 nm. The results were expressed as (+)-catechin equivalents per gram of dry extracts or fractions (mg of CE/g).

2.7 Condensed tannin content determination

Condensed tannin content was determined as reported by Broadhurst and Jones (1978) with a slight modification in our laboratory (Xu & Chang, 2007). All of the procedures were the same except that the volumes of solutions were decreased proportionally. Two-hundred microliters of the mixture were added into a 96-well plate and the absorbance was measured with a plate reader at 500 nm. The content of condensed tannins was expressed as (+)-catechin equivalents per gram of dry extracts or fractions (mg of CE/g).

2.8. Analysis of radical DPPH scavenging activity

DPPH-free scavenging capacity of legume extracts or fractions was determined according to Xu and Chang (2007). All the procedures were the same as Xu and Chang reported before except that the volumes of solutions were decreased proportionally. Two-hundred microliters of the mixture were added into a 96-well plate and the absorbance was measured with a plate reader at 517 nm. The free radical

scavenging activity of the samples was expressed as micromoles of Trolox equivalents per gram **dry extracts or fractions** ($\mu\text{mol TE/g}$).

2.9. Oxygen radical absorbing capacity

A plate reader (Molecular Devices, CA, U.S.A) equipped with adjustable fluorescence filters and incubator was used. The ORAC assay was conducted according to a previous method (Prior et al., 2003; Wu, Beecher, Holden, Haytowitz, Gebhardt, & Prior, 2004) with slight modifications (Xu & Chang, 2007). The ORAC value was calculated and expressed as micromoles of Trolox equivalent per gram of **dry** extracts or fractions ($\mu\text{mol of TE/g}$).

2.10. HPLC analysis of phenolic acids and flavonoids

The quantitative analysis of crude, semi-purified extract and fractions was performed by HPLC according to our previous method (Xu & Chang, 2009) except that an Agilent 1260 infinity quaternary liquid chromatography (Hewlett Packard, Wilmington, NC, USA) was used. All samples were dissolve in 0.1% DMSO aqueous solution and filtered through a 0.2 μm Acrodisc syringe filter (Gelman Laboratory, MI) before injection. Peaks were identified by comparison of retention time and UV spectrum with standards.

2.11. α -Amylase inhibition assay

α -Amylase inhibitory activity was determined using the method of Zhang and coworkers (2011) with a slight modification. Forty microliters of legume extract, fractions or individual **pure** phenolic compounds, 160 μL of distilled water and 400 μL 0.5% starch were mixed in a 1.5 ml centrifuge tube. After adding 200 μL of the enzyme solution (30 unit/ml), the tube was incubated at 25 $^{\circ}\text{C}$ for 3 min. Then, 200 μL of the mixture was taken and added into a separate tube, which contained 100 μL DNS colour reagent solution (96 mM 3,5-dinitrosalicylic acid, 5.31 M sodium

potassium tartrate in 2 M NaOH). The tube **was** placed into a 95 °C thermo mixer (Eppendorf, Hamburg, Germany) for 10 min to inactivate the enzyme. Nine hundred microliters of distilled water **was** added into the tube and mixed well. Then 200 µL of mixture **was** taken and added into a 96-well plate. The absorbance of the reaction mixture was measured at 540 nm. To eliminate the background absorbance produced by legume extracts or fractions, an appropriate extract control without enzyme was included. Type-1 α-amylase inhibitor from *Triticum aestivum* was also analyzed as a positive control. α-Amylase inhibitory activity was measured at five different concentrations, and a logarithmic regression curve was established to calculate IC₅₀ values (mg/ml).

$$\alpha - \text{Amylase inhibition\%} = \left[1 - \left(\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{test}} - A_{\text{control}}} \right) \right] \times 100\%$$

Where A_{sample} is the absorbance of the mixture of extract, starch, enzyme and DNS colour reagent solution; A_{blank} is the absorbance of the mixture of starch solution, extract and DNS colour reagent; A_{test} is the absorbance of the mixture of starch, enzyme and DNS colour reagent; A_{control} is the absorbance of the mixture of starch solution and DNS colour reagent without enzyme.

2.12. α-Glucosidase inhibition assay

α-Glucosidase inhibitory activity was determined according to a previously reported method with slight modifications (Zhang et al., 2011). Commercial α-glucosidase inhibitor voglibose (**a clinical medicine**) was also analyzed as a positive control. In brief, 80 µL of each phenolic extract or fraction with appropriate concentrations **was** mixed with 100 µL of 4 mM 4-nitrophenyl β-D-glucuronide (pNPG) solution (dissolved in 0.1 M pH 6.8 phosphate buffer) in **a** 1.5 ml centrifuge tube, and 20 µl of the 1U/ml enzyme solution **was** added to start the reaction at 37 °C

for 10 min. After 10 min, 200 µl of the reaction mixture was taken and added into a 96-well plate. The release of *p*-nitrophenol from pNPG was measured at 405 nm. The percentage of inhibition was calculated by the equation below. α -Glucosidase inhibitory activity was measured at five different concentrations, and a logarithmic regression curve was established to calculate IC₅₀ values (µg/ml).

$$\alpha - \text{Glucosidase inhibition\%} = \left[1 - \left(\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{test}} - A_{\text{control}}} \right) \right] \times 100\%$$

Where A_{sample} is the absorbance of the mixture of sample and pNPG solution with enzyme; A_{blank} is the absorbance of the mixture of the phenolic sample and pNPG solution without enzyme solution; A_{test} is the absorbance of the mixture of buffer (instead of sample) and pNPG solution with enzyme solution; A_{control} is the absorbance of the mixture of buffer and pNPG solution without enzyme.

2.13. Lipase inhibition assay

The lipase inhibition assay was conducted according to the method described by Zhang and coworkers (2011) with some modifications. Commercial lipase inhibitor URB602 was measured as a positive control. *p*-Nitrophenol palmitate (pNPP) was used as substrate which was hydrolyzed by lipase to *p*-nitrophenol (pNP). In brief, 450 µL 0.05 M sodium phosphate buffer (pH 7.6), containing sodium cholate (1.15 mg/ml) and arabic gum (0.55 mg/ml) was mixed with 50 µL pNPP in isopropanol (0.01 M) and 5 µL of legume extract in a centrifuge tube (1.5 ml size). Five microliters of porcine lipase enzyme solution (50 mg/ml) was added and incubated at 37 °C for exactly 5 min. A portion of 200 µl of mixture was taken and added into a 96-well plate. The absorbance was measured at 410 nm. The percentage inhibition was calculated using the equation below. Lipase inhibitory activity was measured at five different concentrations, and a logarithmic regression curve was

established to calculate IC₅₀ values (mg/ml).

$$\text{Lipase inhibition\%} = \left[1 - \left(\frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{test}} - A_{\text{control}}} \right) \right] \times 100\%$$

Where A_{sample} is the absorbance of the mixture of sample, gum solution and enzyme solution; A_{blank} is the absorbance of the mixture of the phenolic sample and gum solution but without extract; A_{test} is the absorbance of the mixture of buffer, gum solution and enzyme (without sample); A_{control} is the absorbance of the mixture of sample and gum mixture without legume extract or fractions.

2.14. Statistical analysis

Experiments were performed based on a completely randomized design. Each assay was carried out in triplicate. Data were analyzed by ANOVA using 2014 SAS (version 9.3, SAS Inc., Cary, NC, U.S.A.). Duncan's multiple range test was carried out to determine any significant differences between different legume varieties, extracts and fractions ($\alpha=0.05$). Correlation among variables were conducted using Pearson's correlation method with $P < 0.05$ for significance.

3. Results and discussion

3.1. Total phenolic, flavonoid and condensed tannin content

As shown in Table 1, the total phenolic contents of semi-purified extracts from black turtle bean and black soybean were increased from 60.03 mg GAE/g and 40.07 mg GAE/g to 331.43 mg GAE/g and 227.86 mg GAE/g, respectively. After eluting from a Sephadex LH-20 column, the water-eluted fractions (I and II) had lower total phenolic content than Fractions III, IV and V. The highest total phenolic content was found in Fraction IV, containing 599.22 mg GAE/g for black turtle bean and 273.04 mg GAE/g for black soybean, respectively. Fraction I for both beans showed little phenolic compound content. Water-eluted fractions might contain sugar residues or

non-phenolic water-soluble compounds. Most phenolic substances had strong affinity for XAD-7 resin and were not eluted with water. Total phenolic content in black turtle bean was significantly ($P < 0.05$) higher than black soybean for all of the extracts and fractions except Fraction I. The reason might be that the fractions were eluted with different solvents: the last three fractions (III-V) of black soybean were all eluted by 50% ethanol; however, the last fraction (Fraction V) of black turtle bean was eluted by 50% acetone. Thus, the polarity of the last fraction of black turtle bean was lower than that of black soybean, indicating that the last fraction of black turtle bean might possess fewer polar compounds. In addition, the total phenolic content of black turtle bean crude extract was significantly ($P < 0.05$) higher than that of black soybean (Table 1). This might be due to genetic differences, and this point was consistent with our previous study (Xu & Chang, 2007). Overall, XAD-7 column chromatography was effective in removing sugar and organic acids (Zou, Chang, Gu, & Qian, 2011).

Flavonoids are widely present in plants. The consumption of flavonoid-rich foods could protect against human diseases related to oxidative stress (Lotito & Frei, 2006). Flavonoid contents (mg CE/g) in crude extracts, semi-purified extracts and fractions from Sephadex LH-20 column chromatography are shown in Table 1. The TFC in semi-purified extracts was much higher than that in the crude extracts. After Sephadex LH-20 column chromatography, water-eluted fractions, in particular Fraction I, which had a lower flavonoid content in comparison with the last three fractions, similar to the results observed in our previous study (Zou, Chang, Gu, & Qian, 2011) for lentil phenolic substances. For black turtle bean, the highest TFC was found in Fraction V, containing 295.31 mg CE/g, followed by Fraction IV, semi-purified extract and Fraction III in descending order. For black soybean, the highest TFC was found in Fraction V, containing 189.00 mg CE/g, followed by the

semi-purified extract, Fraction III and Fraction IV **in descending order**. However, in our previous study, Fraction IV of lentil from Sephadex LH-20 column was 367.7 mg CE/g (Zou, Chang, Gu, & Qian, 2011), which **was** higher than the respective fractions of black turtle bean and black soybean. Overall, TFC distribution followed a similar pattern to total phenolic content in these two legumes; and black turtle bean had significantly ($P < 0.05$) higher TFC than black soybean for all extracts and fractions except Fraction I.

Condensed tannins are mainly found in the testa of legumes, and can protect them from oxidative damage by some environmental factors (Troszynska & Ciska, 2002). Condensed tannin contents of extracts and fractions from black turtle bean and black soybean are presented in Table 1. Condensed tannins are relatively high molecular weight compounds and can be eluted by acetone. No condensed tannin content was detected in water-eluted Fraction I and II for either **legume**. For black turtle bean, the CTC in Fraction V was the highest (906.32 mg CE/g), and all fractions and semi-purified extracts contained large amounts of condensed tannins. It should be noted that Fraction IV of black soybean contained the highest CTC (797.53 mg CE/g) of all of the fractions, which meant this fraction was mainly composed of condensed tannin or **its constituent monomers**. Using a similar purification method we have previously reported **that** the highest CTC was found in the last fraction (V) of lentil which contained 744.5 mg CE/g, Fraction III only contained 96.5 mg CE/g (Zou, Chang, Gu, & Qian, 2011); and in our small red bean study, the last fraction (V) contained 591.6 mg CE/g (Zou & Chang, 2014). Black turtle bean possessed significantly ($P < 0.05$) higher condensed tannin content than black soybean for all extracts and fractions except Fraction III and IV.

3.2. Antioxidant activity of extractions and fractions

It is not appropriate to evaluate the antioxidant activity with only one single method since antioxidant activity assay methods are based on different mechanisms (Prior, Wu, & Schaich, 2005). Numerous methods have been developed to determine the antioxidant activity, among which DPPH assay and oxygen radical absorbance capacity (ORAC) are the most commonly used methods to evaluate the antioxidant activity of foods. DPPH assay is based on the electron transfer mechanism, in which, when accepting an electron from an antioxidant, DPPH can be reduced to non-radical form from the radical form. ORAC assay depends on a hydrogen transfer mechanism that leads to a decrease in fluorescent intensity. DPPH, ORAC and FRAP assay have recently been used to determine the antioxidant capacity of yellow bean and green bean sprouts in our laboratory. The results indicated that antioxidant capacity patterns for DPPH and FRAP were different to that obtained for ORAC due to a different antioxidative reaction mechanism (Chen & Chang, 2015).

The results from DPPH assays are shown in Table 2. After filtering through XAD-7 column chromatography, the antioxidant activity of semi-purified extracts significantly increased. The water-eluted fractions (I and II) contained much lower antioxidant activity compared to Fractions III, IV and V. Black turtle bean showed significantly ($P < 0.05$) higher DPPH values than black soybean for all of the corresponding extracts and fractions except Fraction I; for black turtle bean, the highest antioxidant activity was found in Fraction V (5001.38 $\mu\text{mol TE/g}$), followed by Fraction IV (4485.54 $\mu\text{mol TE/g}$), Fraction III (2660.56 $\mu\text{mol TE/g}$) and semi-purified extract (2660.56 $\mu\text{mol TE/g}$). For black soybean, Fraction IV rather than Fraction V had the highest antioxidant activity (3751.27 $\mu\text{mol TE/g}$). Fraction V of lentil eluted by Sephadex LH-20 (Zou, Chang, Gu, & Qian, 2011) presented a comparable DPPH scavenging activity (5031.6 $\mu\text{mol TE/g}$) to Fraction V of black

turtle bean.

The ORAC values of extracts and fractions are shown in Table 2. For black turtle bean, Fraction V possessed the highest free-radical scavenging activity followed by Fraction IV (31449.40 $\mu\text{mol TE/g}$) and Fraction III (21538.00 $\mu\text{mol TE/g}$). This pattern was similar to that of the total flavonoid content and condensed tannin content in black turtle bean but different to that of the total phenolic content. However, for black soybean, Fraction IV showed the highest antioxidant activity (31932.14 $\mu\text{mol TE/g}$), followed by Fraction V (27129.27 $\mu\text{mol TE/g}$). Black turtle bean tended to have higher DPPH and ORAC in their respective fractions than black soybean. Correlation analysis of black soybean (Table 2 in supplementary material) indicates that the correlation between CTC and ORAC was the strongest ($r = 0.97$, $P < 0.05$), and Fraction IV exhibited higher condensed tannin content than Fraction V. Therefore, higher condensed tannin content in Fraction IV might contribute to the higher ORAC values of black soybean. For both of the two legumes, Fractions IV and V had higher ORAC values than our previous studies showed for lentil and small red bean (Zou, Chang, Gu, & Qian, 2011; Zou & Chang, 2014).

3.3. HPLC analysis of phenolic acids and flavonoids

The content of individual phenolic acids and flavonoids from black turtle bean and black soybean fractions are shown in Table 3. All of the crude and semi-purified extracts, and 5 fractions from black turtle bean and black soybean were analysed. No phenolic acids or flavonoids were found in water-eluted fractions (I and II). For crude and semi-purified extracts, because of a co-elution problem with other compounds, quantification was not carried out due to impurity. Generally, Fraction III of both legumes contained the most phenolic acids, and Fraction IV of both legumes contained the most flavonoids. All individual phenolic acids and flavonoids in

Fraction III and IV were well separated by HPLC and show high purity factors as determined by the HPLC system. Four phenolic acids in fractions of black turtle bean and 5 phenolic acids in fractions of black soybean were identified. Gallic acid and syringic acid were found in fractions of both black turtle bean and black soybean. However, ferulic acid and sinapic acid were only found in Fraction III of black turtle bean, and *p*-hydroxybenzoic acid and vanillic acid were only found in Fraction III of black soybean. Six flavonoids in fractions of black turtle bean were identified, and 3 flavonoids in fractions of black soybean. Catechin, epicatechin and quercetin-3-O-glucoside were found to be present in fractions of both legumes. However, only myricetin, kaempferol-3-O-rutinoside and kaempferol-3-O-glucoside were found in Fraction III of black turtle bean. In addition, although several peaks were observed in the HPLC analysis of Fraction V of both legumes, they did not correlate with phenolic acid or flavonoid content(Table 1). The peaks in Fraction V of both legumes indicated condensed tannins, since Fraction V is condensed tannin-rich as described in earlier work (Zou, Chang, Gu, & Qian, 2011). It was not possible to analyse the peaks because of a lack of authentic standards for the HPLC analysis. Our interpretation was in accordance with the condensed tannin content observed in Fraction V of both legumes (Table 1). An HPLC-MS-TOF technique will be employed to analyse compounds in Fraction V in our future studies.

3.4. α -Amylase inhibition assay

Managing postprandial plasma glucose levels is important in the early treatment of diabetes (Kim, Jeong, Wang, Lee, & Rhee, 2005). Inhibiting enzymes like α -amylase and α -glucosidase, which are involved in the carbohydrate digestion, is an important method for decreasing postprandial hyperglycemia (Kim, Jeong, Wang, Lee, & Rhee, 2005). The results of the α -amylase inhibition studies are shown in Table 4. All bean

extracts and the last three fractions possessed lower IC₅₀ values (the lower, the more potent) than the commercial inhibitor (Type I from *Triticum aestivum*) under the assay conditions. Water-eluted fractions from Sephadex LH-20 column chromatography showed no significant inhibition despite doses of up to 2 mg/ml in the reaction. Extracts and fractions of black soybean showed significantly ($P < 0.01$) higher inhibitory ability than black turtle bean except for the semi-purified extract.

For black turtle bean, Fraction V possessed the lowest IC₅₀ (0.67 mg/ml), which meant that the last fraction had the highest α -amylase inhibition ability. This might be because the last fraction had the highest condensed tannin content. For black soybean all fractions (except I and II) were more effective than that from black turtle bean; and Fraction V had the highest inhibitory activity (lowest IC₅₀: 0.25 mg/ml) of all fractions against α -amylase. However, the TPC, TFC and CTC values of the last fraction of black turtle bean were higher than those of black soybean, suggesting α -amylase inhibition might be more dependent on individual phenolic compounds than total phenolic compound content or antioxidant activity. In agreement with the literature, Fraction V was mainly composed of condensed tannin (Zou, Chang, Gu, & Qian, 2011). However, condensed tannin content of Fraction V from black soybean was lower than that of black turtle bean. This suggested that the inhibition of α -amylase might be more dependent on some specific condensed tannin structures, or a combination of different condensed tannins might have a synergistic effect. IC₅₀ values of methanolic extract and acetic extract from chokeberry against α -amylase have been reported to be 10.31 mg/ml and 13.55 mg/ml, respectively (Worsztynowicz, Napierala, Bialas, Grajek, & Olkowicz, 2014). Compared to these values, the legume crude extracts, semi-purified extracts and fractions were more effective than chokeberry extract. In general, most literature (Apostolidis & Lee,

2010; Zhang et al., 2011) used crude extracts to conduct an α -amylase inhibition assay without using a commercial inhibitor or pure phenolic compounds as a positive control, and subsequently, this makes comparing our IC₅₀ values with reports in the literature difficult.

3.5. α -Glucosidase inhibition assay

As shown in Table 4, all extracts and fractions except water-eluted fractions (I and II) were more effective inhibitors against α -glucosidase than the α -glucosidase commercial inhibitor (voglibose). All extracts and fractions from black turtle bean possessed a significantly ($P < 0.05$) higher inhibition ability than those from black soybean against α -glucosidase. For both legumes, Fraction V possessed the highest inhibition activity (the lowest IC₅₀ value) followed by Fraction IV and III. The disparity between Factions III and IV could be reflected by the distinct compositions of phenolic acid and flavonoid as determined by HPLC (Table 3). As shown in Table 4, the extracts and fractions of black turtle bean were more effective than the corresponding extracts and fractions from black soybean. For comparison, the IC₅₀ value of voglibose was determined to be 282.13 μ g/ml under our assay conditions. The water-eluted fractions (I and II) showed no significant inhibition activity even using doses up to 1 mg/ml.

Grape skin extract has been reported to have excellent α -glucosidase inhibition activity with an IC₅₀ of 10.5 μ g/ml (Zhang et al., 2011). However, Fraction V of black turtle bean was 42-fold more effective than grape skin extract, and 3.2 fold more effective than Fraction IV of black turtle bean. Even Fraction V of black soybean was 2.5-fold more effective than grape skin extract. To the authors knowledge, Fraction V of black turtle bean is much stronger than oolong tea extract (IC₅₀ = 1340 μ g/ml), green tea extract (IC₅₀ = 7350 μ g/ml) (Oki, Matsui, & Osajima,

1999), or *Barringtonia racemosa* Roxb. Seed extract (26.96 µg/ml) against yeast α-glucosidase (Gowri, Tiwari, Ali, & Rao, 2007). In a recent study, IC₅₀ values of 20 varieties of Canadian lentil phenolic extract were all reported to be higher than 20 mg/ml (Zhang et al., 2015), thereby significantly less effective than any of our extracts or fractions. The current study suggests that natural α-amylase and α-glucosidase inhibitors from black legumes provide a potential strategy to control post-prandial hyperglycemia. Inhibitors from food sources with lower inhibitory ability against α-amylase and stronger inhibition of α-glucosidase can minimize side effects such as abdominal distention and flatulence (Kown, Apostolidis, & Shetty, 2008). Our findings show that black legumes have this potential and may reduce these side effects of starch digestion.

3.6. Lipase inhibition assay

As shown in Table 4, all extracts and fractions except water-eluted fractions were effective inhibitors against pancreatic lipase *in vitro* compared with commercial pancreatic lipase inhibitor (URB602). For both legumes, Fraction IV possessed the lowest IC₅₀ values (0.076 mg/ml for black turtle bean and 0.081 mg/ml for black soybean) and these were similar to the commercial inhibitor (0.083 mg/ml). The water-eluted fractions (I and II) showed no significant inhibition activity even with doses up to 0.5 mg/ml in the reaction. Fraction IV was more effective than Fraction V for both legumes and this might be because flavonoids have a higher lipase inhibition activity than tannins and this assumption is in accordance with the observation that punicalagin (tannin) showed a higher IC₅₀ value (125 µM) than other flavonoid standards (less than 100 µM) (Sergent et al., 2012).

It has been reported that tannin-rich berry extract possesses high lipase inhibition activity (McDougall, Kulkarni, & Stewart, 2009). Researchers attributed

this inhibition activity to tannin structures, which display lipase binding affinity (Sugiyama et al., 2007). Fraction V of black turtle bean showed the highest condensed tannin content. However it did not exhibit the lowest IC₅₀ value, suggesting that condensed tannin content was not the only factor for the outstanding lipase inhibition activity. It is likely that some specific structures were more effective than others for lipase inhibition. Therefore, the amount of condensed tannin alone might not be the best indicator for lipase inhibition ability. A combination of other phenolic compounds and tannins might have some synergistic effect since our Fraction IV had the highest total phenolic content. Epigallocatechin-3-O-gallate (EGCG) was one of the major polyphenols in green tea, showing lipase inhibition with an IC₅₀ of 0.349 µM (0.159 mg/ml equivalent) (Nakai et al., 2005). Recently, crude phenolic extracts from 20 varieties of lentil were used to determine IC₅₀ values of lipase inhibition, and the results showed that the IC₅₀ values of all of these phenolic extracts against lipase were higher than 6 mg/ml (Zhang et al., 2015), which were significantly higher than the values obtained in this study. However, they did not use any commercial inhibitors as positive control. Therefore, it is difficult to fairly compare their IC₅₀ values with the results from the present study, which arose from different assay conditions and plant materials.

3.7. The inhibition of α-amylase, α-glucosidase and lipase by commercial pure phenolic compounds.

Black legumes are phenolic-rich material. In our previous studies (Xu & Chang, 2008; 2009), we reported that gallic acid, vanillic acid, caffeic acid, 2,3,4-trihydroxybenzoic acid, sinapic acid, myricetin, chlorogenic acid, salicylic acid and syringic acid were the major phenolic acids and flavonoids in black turtle bean and black soybean. In this study, in addition to the phenolic compounds reported

before, protocatechuic acid, p-hydroxybenzoic acid, ferulic acid, catechin, epicatechin, quercetin-3-O-glucoside, kaempferol-3-O-rutinoside and kaempferol-3-O-glucoside were discovered. Therefore, these HPLC-grade phenolic compounds were used to test α -amylase, α -glucosidase and lipase inhibition activity. Results are presented in Table 5. All of the tested compounds had affected the inhibition of the three digestive enzymes except for salicylic acid, which showed no inhibition of α -glucosidase. However, the ability of the selected phenolic compounds varied significantly ($P < 0.05$).

For α -amylase inhibition, myricetin had the highest inhibition activity with IC_{50} values ($1.19 \text{ mM} = 0.38 \text{ mg/ml}$) lower than the commercial α -amylase inhibitor (3.23 mg/ml). For α -glucosidase inhibition activity, myricetin also showed the lowest IC_{50} value ($0.87 \text{ }\mu\text{g/ml} = 2.73 \text{ }\mu\text{M}$), followed by kaempferol-3-O-rutinoside ($6.05 \text{ }\mu\text{g/ml} = 10.13 \text{ }\mu\text{M}$) and epicatechin ($3.41 \text{ }\mu\text{g/ml} = 11.75 \text{ }\mu\text{M}$). All phenolic standards tested had IC_{50} values lower than that of the commercial inhibitor ($281.22 \text{ }\mu\text{g/ml}$). In the literature, caffeic acid, coumaric acid, gallic acid and quercetin (1 mg/ml) were tested for α -glucosidase inhibition, but very low inhibitory activity was observed (Kwon, Apostolidis, & Shetty, 2008). Caffeic acid showed much lower IC_{50} ($40.23 \text{ }\mu\text{g/ml}$) value in our study. This reason might be because Kwon et al. did not adjust the pH of the phenolic standard, since the inhibition rate increased significantly after pH adjustment to 6.5-7.2. In another study, pure phenolic standards (catechin, epicatechin, kaempferol, quercetin and some derivatives) were used to determine the IC_{50} values against α -glucosidase, and quercetin-arabinoside was found to possess the highest inhibition activity ($IC_{50} = 80.28 \text{ }\mu\text{g/ml} = 0.18 \text{ mM}$) (Zhang et al., 2015). This value was higher than the IC_{50} value of myricetin ($0.87 \text{ }\mu\text{g/ml} = 2.73 \text{ }\mu\text{M}$) obtained in this study. In a lipase inhibition study, myricetin also showed the lowest IC_{50} value (0.015

mg/ml = 0.047 mM), even lower than the commercial inhibitor (0.083 mg/ml= 0.28 mM). Of all of the pure phenolic standards tested, each flavonoid compound showed a lower IC₅₀ than the commercial inhibitor in terms of α -glucosidase inhibition. In the Canadian lentil study mentioned above (Zhang et al., 2015), quercetin-arabinoside showed the lowest IC₅₀ value against lipase (20.81 μ g/ml), which was 27.9% higher than the IC₅₀ value of myricetin (15 μ g/ml) determined in this study.

Our study is the first to show that myricetin, naturally present in legumes (Xu & Chang, 2009), is a highly potent enzyme inhibitor of the three main digestive enzymes. It has been demonstrated for the first time that seventeen phenolic compounds (11 phenolic acids and 6 flavonoids) show inhibition of α -amylase, α -glucosidase and lipase. Generally, our results show that flavonoids have higher enzyme inhibition activity than phenolic acids especially with regards to α -glucosidase. It has been reported that enzymes belonging to the glycoside hydrolase family 13, such as α -amylase and α -glucosidase, share a common inhibition reaction mechanism (Zhang et al., 2011). However, salicylic acid showed significant inhibition of α -amylase but not α -glucosidase, suggesting that the inhibitory reaction mechanisms of salicylic acid against α -glucosidase and α -amylase are not the same. Generally, phenolic compounds have the ability to bind to digestive enzymes to alter their activities. In one study, the phenolic extracts from Pontal and Pinto beans showed α -amylase inhibition activity with inhibition rates ranging from 25.8% to 74.2%, and myricetin was found in the extracts using LC-ESI-MS (Mojica, Meyer, Berhow, & Mejia, 2015). However, it was not possible to fairly compare their results with our study since these authors did not use the pure inhibitor as a reference in their study and inhibition activity was presented as percentage instead of IC₅₀ value.

A recent study showed myricetin could significantly inhibit the differentiation

of 3T3-L1 cell from pre-adipocytes into adipocytes at 50 μ M (Wang, Wang, Yang, You, & Zhang, 2015), suggesting myricetin had anti-obesity activity. In addition, myricetin had the function to increase the sensitivity of insulin (Liu, Tzeng, Liou, & Lan, 2007) through improving impaired signaling intermediates of insulin receptors (Tzeng, Liou, & Liu, 2011). Our study and others show that myricetin could be a potential phenolic compound for treating postprandial hyperglycemia and obesity. Myricetin could be increased by breeding or genetic manipulation methods for enhancing enzymatic inhibition by legumes for the management of diabetes.

3.8. Pearson correlation coefficient analysis

Many previous studies have indicated that total phenolic content is one of the important parameters for antioxidant activity (Ismail, Marjan, & Foong, 2004; Xu, Yuan, & Chang, 2007). Therefore, Pearson correlation analysis was conducted to analyze the correlation between antioxidant activity, phenolic substances and enzyme inhibition ability. For black turtle bean, significant correlations were found among all types of phenolic contents and enzyme inhibition ability (Table 1 in supplementary material). The strongest correlation was found between TPC and lipase inhibition ability ($r = -0.96$, $P < 0.01$). Comparison of coefficients among antioxidant activity and phenolic contents revealed that significant correlations existed between all of the parameters. Condensed tannin content correlated with DPPH and ORAC the strongest, with $r = 0.98$ and $r = 0.96$, respectively. In terms of α -amylase inhibition activity, the correlation coefficient between total flavonoid content and α -amylase inhibition activity was the strongest ($r = -0.93$, $P < 0.05$). As to α -glucosidase inhibition activity, correlation between DPPH and α -glucosidase inhibition activity was the strongest ($r = -0.95$, $P < 0.05$). However, the coefficient between ORAC and α -glucosidase

inhibition activity ($r = -0.78$) was lower than that between DPPH and α -glucosidase inhibition activity ($r = -0.95$).

For black soybean, significant correlations only existed between some parameters, but not between TPC and CTC, TFC and CTC, TFC and lipase inhibition activity, CTC and α -glucosidase inhibition activity, and ORAC and α -glucosidase inhibition ability (Table 2 in supplementary material). The correlation between CTC and DPPH and ORAC were 0.96 and 0.97 ($P < 0.01$), respectively. No significant correlations were observed between TPC, TFC and lipase inhibition ability. However, pure phenolic acids and flavonoids were used to test the inhibition activity against three digestive enzymes and all of them showed inhibition of those three enzymes to a different extent (except that salicylic acid shows no inhibition of α -glucosidase).

Strictly speaking, correlations cannot prove a cause-effect relationship between phenolic substances content, antioxidant activities and enzymes inhibition since the phenolic substance extracts or fractions were not pure single compounds and a combination of some specific structures might have a synergistic effect or antagonistic effect. In addition, more research at the molecular level is necessary to understand the reaction mechanisms of enzyme inhibition by legume phenolics.

4. Conclusion

In comparison to commercial inhibitors, several fractions of both black turtle bean and black soybean obtained from Sephadex LH-20 column fractionation were more effective inhibitors against α -amylase, α -glucosidase and lipase. The crude extract and three major column fractions of black soybean showed a higher inhibitory capacity against α -amylase. However, both crude and semi-purified extracts and three major column fractions of black turtle bean showed a superior inhibitory capacity against α -glucosidase. These two legumes showed similar inhibitory capacity against

lipase. Among the pure phenolic compounds studied, myricetin, a flavonoid present in legumes, was the most potent for suppressing activity of α -amylase and lipase but with no significant difference from other flavonoids as to inhibition of α -glucosidase. This study contributes to the understanding of the potential effectiveness in the use of the two black legumes for the management of diabetes. However, since legumes need to be cooked prior to consumption, the retention of the phenolic compounds by cooking legumes should be studied in the future. Nonetheless, the major column-isolated fractions, which exhibited high enzyme inhibition capabilities, may be developed into dietary supplement products for the management of the digestion of starch and lipids for improving the health of people who may benefit from the reduced digestion of these food components.

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708

1 Table 1. TPC, TFC, CTC of extracts and fractions from black turtle bean and black soybean.

	Total Phenolic Content (mg GAE/g)		Total Flavonoid content (mg CE/g)		Condensed Tannin Content (mg CE/g)		Yield (%)	
	Black turtle bean	Black soybean	Black turtle bean	Black soybean	Black turtle bean	Black soybean	Black turtle bean	Black soybean
Crude extract	60.03±0.28f*	40.07±0.14e	70.21±0.53e*	49.64±0.31e	40.69±0.75e*	20.64±0.12e	9.45%	9.16%
Semi-purified extract	331.43±16.16d*	227.86±10.01b	174.78±0.51c*	139.78±1.52b	354.75±22.91d*	187.38±7.95d	8.86%	8.56%
Fraction I	15.28±0.49g	17.96±1.41f*	15.31±3.09g	19.28±1.37f*	ND	ND	4.5%	3.2%
Fraction II	75.66±1.13e*	58.16±2.68d	59.38±0.88f	61.31±2.13d	ND	ND	2.7%	5.2%
Fraction III	363.20±2.83c*	144.64±5.06c	143.33±1.44d*	139.25±1.06b	571.21±14.14c	670.50±70.70b*	2.1%	1.3%
Fraction IV	599.22±2.83a*	273.04±1.13a	281.25±3.54b*	110.75±1.96c	691.34±19.41b	797.53±42.43a*	3.7%	2.3%
Fraction V	481.21±16.97b*	225.44±1.69b	295.31±2.21a*	189.00±0.71a	906.32±63.64a*	600.51±7.07c	42.1%	43%

2 Data are expressed as mean ± standard deviation (n=3); values marked by the different letter within the same column are significantly different
3 ($P < 0.05$) among different extracts and fractions. Values marked by asterisk were significantly different ($P < 0.05$) between two varieties in the
4 same extracts or fraction type. All data were based on dry weight basis.

5 Table 2. DPPH and ORAC of extracts and fractions from black turtle bean and black soybean.

	DPPH Value ($\mu\text{mol TE/g}$)		ORAC Value ($\mu\text{mol TE/g}$)	
	Black turtle bean	Black soybean	Black turtle bean	Black soybean
Crude extract	49.25 $\pm$ 1.35f*	23.77 $\pm$ 0.62g	80.02 $\pm$ 3.94g	100.76 $\pm$ 4.41g*
Semi-purified extract	2660.56 $\pm$ 68.56d*	1572.77 $\pm$ 38.34d	3800.2 $\pm$ 124.04d	4853.67 $\pm$ 100.26d*
Fraction I	51.15 $\pm$ 1.28f	47.35 $\pm$ 3.82f	1805.56 $\pm$ 159.49f*	1121.42 $\pm$ 89.24f
Fraction II	185.09 $\pm$ 5.62e*	152.09 $\pm$ 3.61e	2678.28 $\pm$ 159.18e*	1789.25 $\pm$ 128.31e
Fraction III	3263.82 $\pm$ 51.19c*	2622.48 $\pm$ 32.14c	21538.00 $\pm$ 91.63c*	19520.80 $\pm$ 285.41c
Fraction IV	4485.54 $\pm$ 12.83b*	3751.27 $\pm$ 21.43a	31449.40 $\pm$ 294.42a	31932.14 $\pm$ 1996.82a*
Fraction V	5001.38 $\pm$ 25.66a*	2978.55 $\pm$ 128.56b	35830.26 $\pm$ 132.61b*	27129.27 $\pm$ 1411.42b

6 Data are expressed as mean $\pm$ standard deviation (n=3); values within each type of sample marked by the different letter within same column are
7 significantly different ($P < 0.05$); Values of DPPH and ORAC within each type of extract or fraction marked by asterisk within the same row are
8 significantly different ($P < 0.05$). All data were based on dry weight basis.

9 Table 3. Phenolic acids and flavonoids composition of fractions from black turtle bean and black soybean.

Phenolic acid	Black turtle bean			Black soybean		
	Fraction 3 ($\mu\text{g/g}$)	Fraction 4 ($\mu\text{g/g}$)	Fraction 5 ($\mu\text{g/g}$)	Fraction 3 ($\mu\text{g/g}$)	Fraction 4 ($\mu\text{g/g}$)	Fraction 5 ($\mu\text{g/g}$)
Gallic acid	2644.46 $\pm$ 13.40b	ND	ND	179.37 $\pm$ 0.66d	ND	ND
Protocatechuic acid	ND	ND	ND	5017.10 $\pm$ 7.52a	ND	ND
Ferulic acid	3021.88 $\pm$ 3.65a	ND	ND	ND	ND	ND
Sinapic acid	641.07 $\pm$ 44.52c	ND	ND	ND	ND	ND
Syringic acid	ND	787.66 $\pm$ 11.43a	ND	ND	14683.82 $\pm$ 27.55a	ND
p-Hydroxybenzoic acid	ND	ND	ND	1240.68 $\pm$ 1.85c	ND	ND
Vanillic acid	ND	ND	ND	2387.80 $\pm$ 40.27b	ND	ND
Flavonoids	Fraction 3 (mg/g)	Fraction 4 (mg/g)	Fraction 5 (mg/g)	Fraction 3 (mg/g)	Fraction 4 (mg/g)	Fraction 5 (mg/g)
Myricetin	2.75 $\pm$ 0.002b	ND	ND	ND	ND	ND
Catechin	ND	107.38 $\pm$ 0.75a	ND	ND	20.25 $\pm$ 0.47a	ND
Epicatechin	ND	93.16 $\pm$ 0.15b	ND	20.83 $\pm$ 0.52a	ND	ND

Table 3. Continued.

Quercetin-3-o-glucoside	ND	23.78±0.04c	ND	ND	12.02±0.10b	ND
Kaempferol-3-o-rutinoside	5.20±0.01a	ND	ND	ND	ND	ND
Kaempferol-3-o-glucoside	ND	19.62±0.04d	ND	ND	ND	ND

Results were expressed as mean ± standard deviation (n = 3) on a dry weight basis. Values marked by the same letter within each fraction in each column are not significantly different ($P < 0.05$), ND = not detectable.

13 Table 4. IC₅₀ values of extracts and fractions from black turtle bean and black soybean against α -amylase, α -glucosidase and lipase.

	IC ₅₀ values (mg/mL) against α -amylase		IC ₅₀ values (μ g/mL) against α -glucosidase		IC ₅₀ values (mg/mL) against lipase	
	Black turtle bean	Black soybean	Black turtle bean	Black soybean	Black turtle bean	Black soybean
Crude extract	2.69 $\pm$ 0.12b*	2.25 $\pm$ 0.011b	64.12 $\pm$ 2.12b	75.41 $\pm$ 3.11b*	0.38 $\pm$ 0.02a*	0.27 $\pm$ 0.011a
Semi-purified extract	1.12 $\pm$ 0.09d	1.60 $\pm$ 0.008c*	13.81 $\pm$ 0.83c	19.52 $\pm$ 1.08c*	0.30 $\pm$ 0.014b*	0.25 $\pm$ 0.008a
Fraction I	>2	>2	>1000	>1000	>0.5	>0.5
Fraction II	>2	>2	>1000	>1000	>0.5	>0.5
Fraction III	1.76 $\pm$ 0.06c*	1.12 $\pm$ 0.03d	8.03 $\pm$ 0.46d	25.01 $\pm$ 1.33d*	0.26 $\pm$ 0.013c*	0.21 $\pm$ 0.009b
Fraction IV	0.96 $\pm$ 0.03e*	0.48 $\pm$ 0.02e	3.28 $\pm$ 0.13e	13.92 $\pm$ 1.02e*	0.076 $\pm$ 0.006e	0.081 $\pm$ 0.009c*
Fraction V	0.67 $\pm$ 0.07f*	0.25 $\pm$ 0.05f	0.25 $\pm$ 0.07f	5.41 $\pm$ 0.045f*	0.17 $\pm$ 0.012d	0.19 $\pm$ 0.015b*
Commercial inhibitor	3.23 $\pm$ 0.21a		281.22 $\pm$ 12.21a		0.083 $\pm$ 0.005e	

14 Results were expressed as mean $\pm$ standard deviation (n = 3), values with different letters within a column were significantly different ($P <$
15 0.05); values within each type of extract or fraction marked by asterisk within the same row are significantly different ($P <$ 0.05). Commercial
16 inhibitor for α -amylase is Type I α -amylase from *Triticum aestivum*; α -glucosidase inhibitor is voglibose (mw: 267.28); lipase inhibitor is
17 URB602 (mw:295.38). All data were based on dry weight basis.

18 Table 5. IC₅₀ values of pure phenolic compounds against α -amylase, α -glucosidase and lipase.

	IC ₅₀ values against α -amylase		IC ₅₀ values against α -glucosidase		IC ₅₀ values against lipase	
	mg/mL	mM	μ g/mL	μ M	mg/mL	mM
Gallic Acid	1.27 $\pm$ 0.04k	7.46 $\pm$ 0.24kj	50.39 $\pm$ 3.00e	296.20 $\pm$ 17.63e	0.86 $\pm$ 0.04lde	5.05 $\pm$ 0.24e
Vanillic Acid	4.69 $\pm$ 0.21f	27.89 $\pm$ 1.24e	46.64 $\pm$ 2.70fg	277.38 $\pm$ 16.06ef	1.49 $\pm$ 0.09a	8.86 $\pm$ 0.53b
Caffeic Acid	1.20 $\pm$ 0.09k	6.66 $\pm$ 0.49k	40.23 $\pm$ 2.50g	223.30 $\pm$ 13.87gh	0.94 $\pm$ 0.09cd	5.22 $\pm$ 0.50e
2,3,4-Trihydroxybenzoic acid	5.67 $\pm$ 0.29d	33.33 $\pm$ 1.70bc	32.16 $\pm$ 1.70h	189.04 $\pm$ 9.99h	1.31 $\pm$ 0.10b	7.70 $\pm$ 0.59c
Sinapic Acid	7.24 $\pm$ 0.41b	32.29 $\pm$ 1.82cd	56.24 $\pm$ 4.30e	250.84 $\pm$ 19.18fg	0.33 $\pm$ 0.03g	1.47 $\pm$ 0.13g
Chlorogenic Acid	4.10 $\pm$ 0.13g	11.57 $\pm$ 0.37gh	82.13 $\pm$ 6.10b	231.80 $\pm$ 17.21g	1.35 $\pm$ 0.09b	3.81 $\pm$ 0.25f
Salicylic Acid	4.72 $\pm$ 0.18f	34.17 $\pm$ 1.30b	ND	ND	1.50 $\pm$ 0.09a	10.86 $\pm$ 0.65a
Syringic Acid	8.88 $\pm$ 0.51a	44.81 $\pm$ 2.57a	73.28 $\pm$ 5.10c	369.78 $\pm$ 25.73d	1.54 $\pm$ 0.11a	7.77 $\pm$ 0.56c
Protocatechuic acid	1.78 $\pm$ 0.07j	11.54 $\pm$ 0.45gh	63.37 $\pm$ 4.10d	411.17 $\pm$ 26.60c	1.03 $\pm$ 0.09c	6.68 $\pm$ 0.58d
p-Hydroxybenzoic acid	1.94 $\pm$ 0.09j	14.04 $\pm$ 0.65f	58.68 $\pm$ 4.20d	424.84 $\pm$ 30.40c	1.25 $\pm$ 0.11b	9.05 $\pm$ 0.80b
Ferulic acid	6.11 $\pm$ 0.25c	31.46 $\pm$ 1.29d	89.47 $\pm$ 6.70b	460.76 $\pm$ 34.50b	0.98 $\pm$ 0.08cd	5.05 $\pm$ 0.41e
Myricetin	0.38 $\pm$ 0.02l	1.19 $\pm$ 0.06l	0.87 $\pm$ 0.03i	2.73 $\pm$ 0.09i	0.015 $\pm$ 0.004h	0.047 $\pm$ 0.003h

Table 5. Continued.

Catechin	2.44±0.11i	8.41±0.38jk	3.89±0.12i	13.40±0.41i	0.53±0.05f	1.83±0.17g
epicatechin	2.13±0.06ij	7.34±0.21jk	3.41±0.14i	11.75±0.48i	0.49±0.04f	1.69±0.14g
Quercetin-3-O-glucoside	4.87±0.28ef	10.48±0.60hi	5.73±0.35i	12.34±0.75i	0.81±0.07e	1.74±0.15g
Kaempferol-3-O-rutinoside	5.15±0.37e	8.66±0.62ij	6.05±0.46i	10.18±0.77i	0.95±0.08cd	1.60±0.13g
Kaempferol-3-O-glucoside	5.57±0.46d	12.42±1.03fg	7.13±0.68i	15.90±1.51i	1.58±0.13a	3.52±0.29g
Commercial Inhibitor	3.23±0.16h	NA	281.22±13.00a	1052.16±48.63a	0.083±0.01h	0.28±0.017h

19 Results were expressed as mean ± standard deviation (n = 3), values with different letters within a column were significantly different ($P <$
20 0.05). Commercial inhibitor for α -amylase is Type I α -amylase from *Triticum aestivum* (protein mixture); α -glucosidase inhibitor is voglibose
21 (mw: 267.28); lipase inhibitor is URB602 (mw:295.38). All data were based on dry weight basis.

Figure 1. Flow chart for the fractionations of phenolic substances extracted from black soybean (A) and black turtle bean (B)