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A Comparative Study on Phenolic Substances in Selected Black Legumes that Inhibit Digestive Enzymes

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By

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A comparative study on phenolic substances in selected black legumes that inhibit digestive enzymes

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Antioxidant-rich plant foods can inhibit starch and lipid digestion that are relevant to the management of type-II diabetes. Our objective was to compare the three phenolic substances (total phenolic, total flavonoids, and condensed tannin content) in crude, semi-purified extracts from eight types of foods (purified by XAD-7 column), five fractions (semi-purified extracts fractionated by Sephadex LH-20 column) from black legumes, and to compare their antioxidant capacities. The IC_{50} values of these crude extracts, semi-purified extracts and fractions against alpha-amylase, alpha-glucosidase and lipase were measured. Results showed that Fraction V from black soybean had the lowest IC_{50} value (0.25 mg/mL) against alpha-amylase; Fraction V from black bean had the lowest IC_{50} value (0.25 micro gram/mL) against alpha-glucosidase; Fraction IV of black bean had the lowest IC_{50} value (76 micro gram/mL) against lipase; myricetin showed the lowest IC_{50} value against alpha-amylase, alpha-glucosidase and lipase.

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

DEDICATION

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CHAPTER I

INTRODUCTION

The health of people is more and more endangered by type-II diabetes. The prevalence of diabetes has been rising at a startling rate. Diabetes currently affects 371 million people worldwide and this number is projected to double by 2030 (Alberti and Zimmet 2013). Diabetes is partly caused by excessive presence of carbohydrates in the diet. Starch digestion of mammalians mainly occurs in the lumen of the small intestine by α-amylase to yield maltose and branched isomaltose oligosaccharides, both of which cannot be absorbed into the bloodstream without further processing (Rossi and others 2006). Inhibition of digestive enzymes or glucose transporters can suppress postprandial hyperglycemia through reduction the rate of glucose release and absorption in the small intestine (Hanhineva and others 2010). In addition, some other reasons also cause the prevalence of type-II diabetes, such as fat-enriched diet and sedentary lifestyle (Cani and others 2008). No doubt, lipids are an essential component in human diet, and hyperglycemia partly results from high intake of lipids (Kopelman 2000). Pancreatic lipase can break down triglycerides to monoglycerides and two fatty acids (Winkler and others 1990).

Some epidemiological and interventional studies indicated that the consumption of phenolic-rich foods is inversely correlated with the prevalence of type-II diabetes (Cieślik and others 2006). Apart from antioxidant capacity, growing evidence indicates

that polyphenol contained in berries, vegetables, nuts and tea possessed many health promoting and disease preventing properties (Roopchand and others 2013). Phenolics can inactivate α-amylase, α-glucosidase and lipase through non-specific binding to enzymes (Zhang and others 2015). Most reported studies used crude phenolic extracts, and the major contents of crude phenolic extracts are sugar and organic acids. However, none of them used purified or semi-purified phenolic compounds, which are isolated from common foods. Therefore, using the semi-purified and purified phenolic compounds to do further analysis is needed. Our objective was to compare three phenolic substances (total phenolic content, total flavonoids, and condensed tannin) in crude, semi-purified extracts (filter through XAD-7 column) and fractions (semi-purified extracts separated through Sephadex LH-20 column) isolated from black bean (also named black turtle bean, *Phaseolus vulgaris* L.), black soybean (*Glycine max* L.), and to compare their antioxidant capacities and inhibitory effects on α-amylase, α-glucosidase and lipase were investigated.

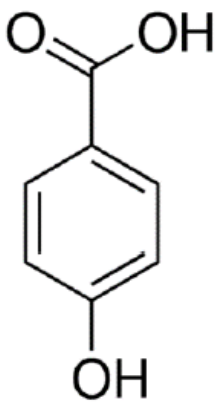
CHAPTER II

LITERATURE REVIEW

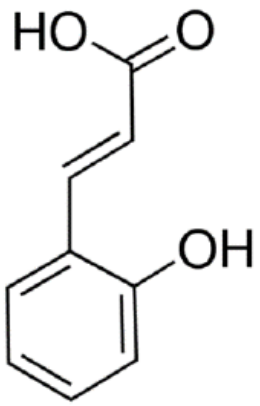
2.1 An overview of plant phenolic compounds

It is well-known that phenolic compounds are very complicated phytochemicals found in almost every plant. Over the last 30 years, people focused on the extraction, identification and quantification of phenolic compounds from plants for both medicinal and dietary composition. Phenolic compounds consist of simple phenol, benzoic and cinnamic acid, tannins, coumarins, lignins, lignans and flavonoids. Organic solvent extraction is one of the popular methods to extract phenolics from plant. Various protocols have been developed to determine the total phenolics, total flavonoids and total condensed tannins. Meanwhile, spectrophotometric and chromatographic techniques are applied to identify and quantify unique phenolic compounds (Khoddami and others 2013).

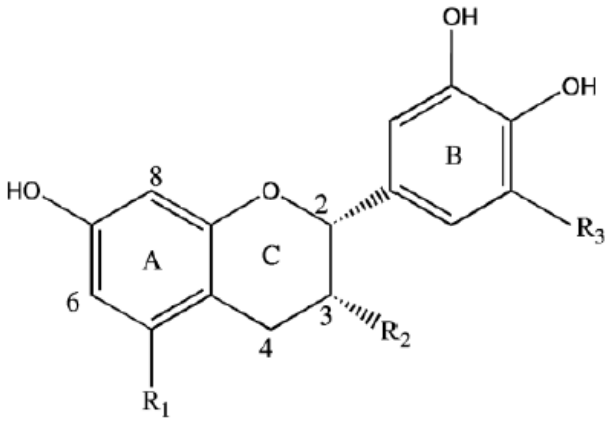
Phenolic compounds are synthesized in plants partly due to ecological and physiological pressures such as UV radiation, wounding, insect attack and pathogens (Kennedy and Wightman 2011; Napal and others 2010). An aromatic ring bearing one or more hydroxyl groups is the basic structure of phenolic compounds. Figure 2.1 shows some elementary structures of phenolic compounds (Soto-Vaca and others 2012).



Hydroxybenzoic acids



Hydroxycinnamic acids



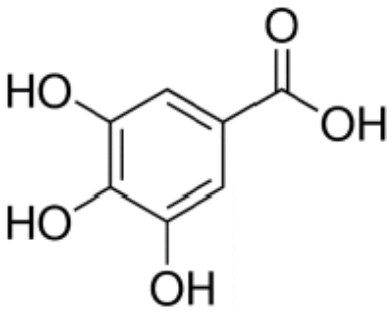
Basic repeating unit in condensed tannin

Figure 2.1 Structures of some elementary phenolic compounds

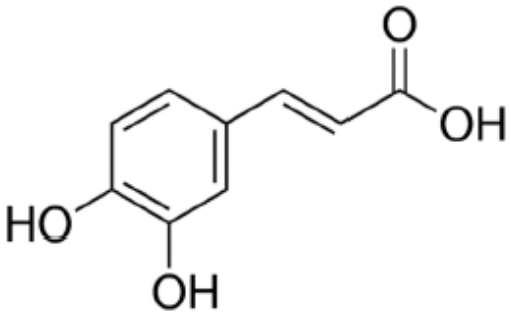
There are no standardized classification methods for plant phenolic compounds. Some people classify plant phenolic into non-flavonoids and flavonoids. The non-flavonoid class is mainly simpler molecules, including derivatives of benzoic acid, cinnamic acid and stilbene (Soto-Vaca and others 2012). The other way is to classify the

plant phenolic compounds into simple phenols and polyphenols according to the number of phenol units in the molecule (Khoddami and others 2013).

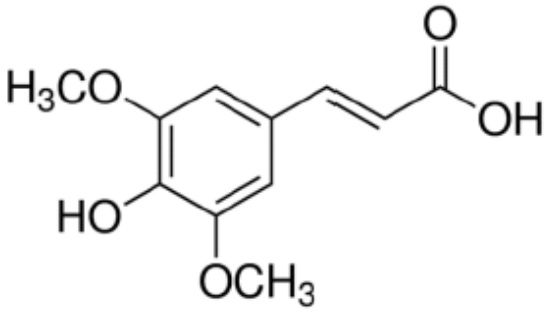
Phenolic acids are the most common phenolic compounds in plants but are rarely present in free forms. Different numbers of hydroxyl groups on the aromatic ring can form hundreds of phenolic acids (Wojdyło and others 2007). Figure 2.2 shows some structures of phenolic acids commonly occurring in plants. Flavonoids are the largest group of plant phenolic compounds, which include at least 2000 naturally occurring compounds in plants, and are widely distributed in plant tissue and often related to the color of plants. Normally, the darker the color, the higher the flavonoid content (McGhie and Walton 2007). Figure 2.3 displays the structures of some common flavonoids.



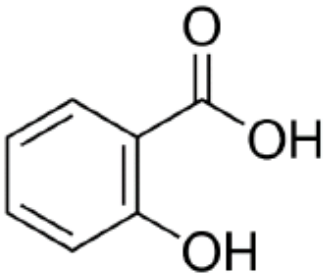
Gallic acid



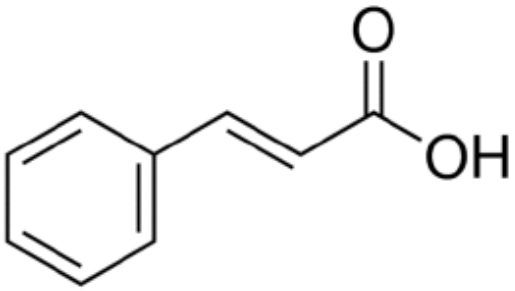
Caffeic acid



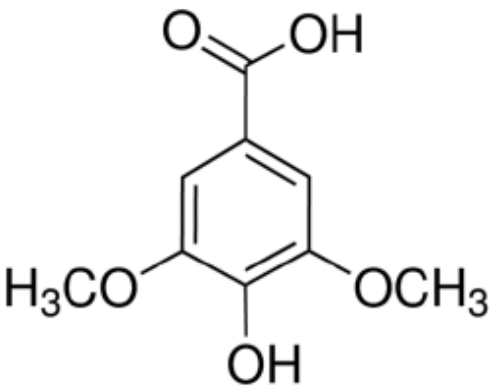
Sinapic acid



Salicylic acid

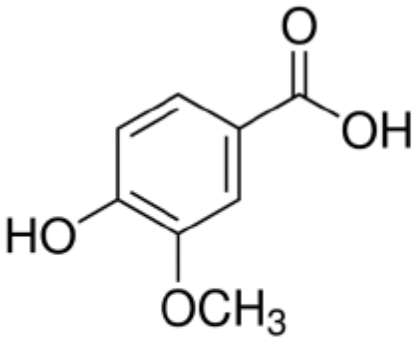


Trans- cinnamic acid

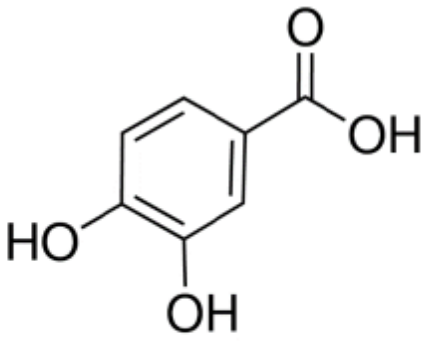


Syringic acid

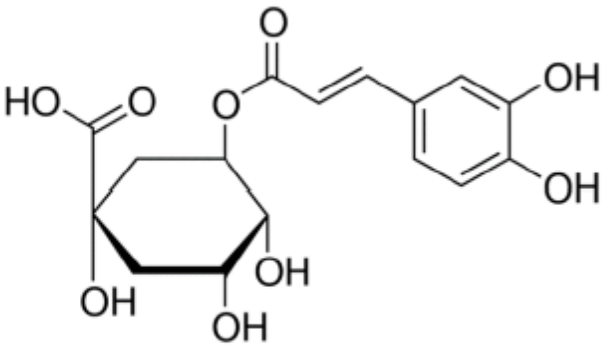
Figure 2.2 Structures of some phenolic acids



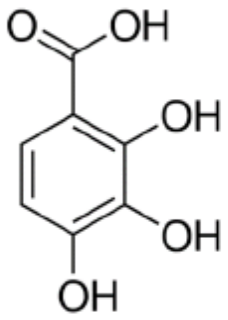
Vanillic acid



3,4-Dihydroxybenzoic acid

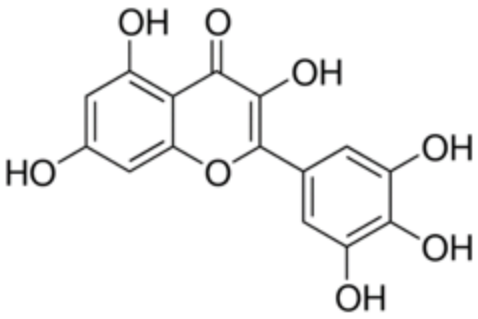


Chlorogenic acid

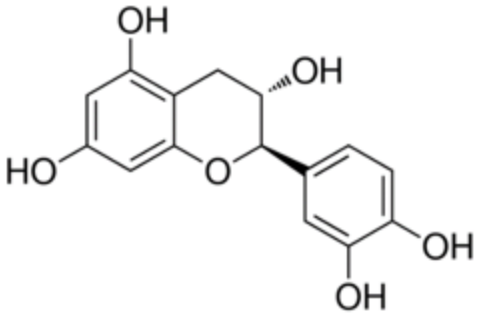


2,3,4-Trihydroxybenzoic acid

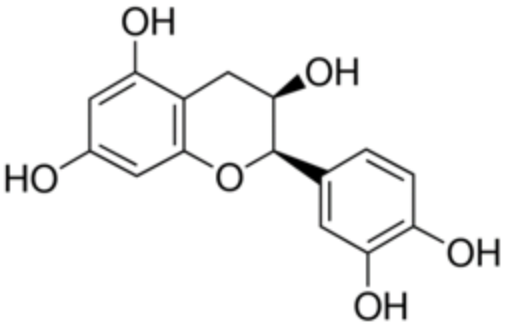
Figure 2.2 (Continued)



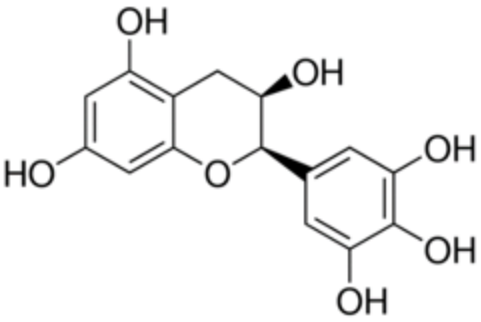
Myricetin



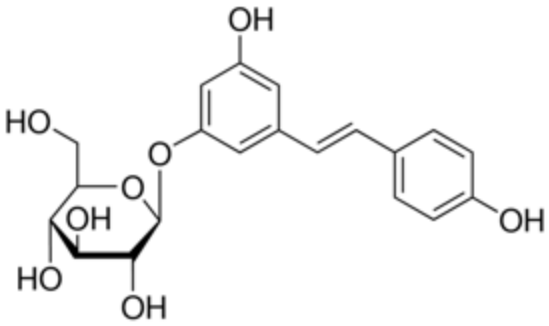
Catechin



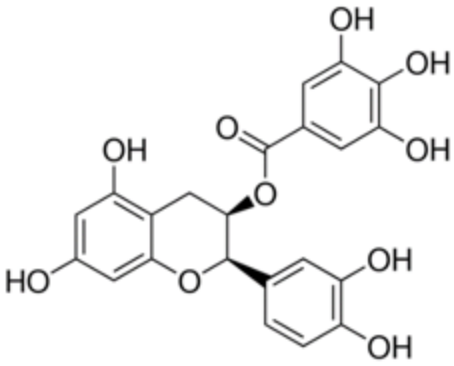
(-)-Epicatechin



(-)-Epigallocatechin

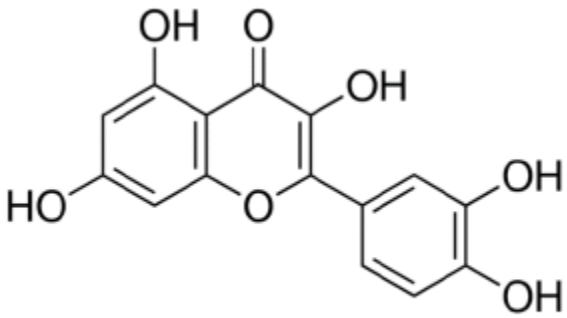


Polydatin



Epicatechin Gallate

Figure 2.3 Structures of some flavonoids



Quercetin

Figure 2.3 (Continued)

2.2 Phenolic-rich foods

Green tea is widely consumed in Asian countries while black tea in West countries (Crespy and Williamson 2004). During the manufacture of black tea, catechins in fresh tea are oxidized by polyphenol oxidase into quinones, which condense to generate theaflavins and thearubigins. However, this process is inactivated by steam or pan heating treatment to produce green tea (Harbowy and others 1997). Catechin is the main phenolic compound in both black and green tea. Catechin content in green tea is 80-90%; however, only 20-30% of catechin are present in black tea. Theaflavins and thearubigins represent about 50-60% of total flavonoids in black tea. Tea and tea extracts possess many health promoting effects such as anti-obesity, anti-diabetes and anti-hypertensive activities (Ali and others 2015; Deng and others 2015; Gostner and others 2015; Xu and others 2015). Many of the above mentioned benefits are related to catechin, especially (-)-epigallocatechin-3-gallate (EGCG) content (Zhang and others 2010). Epidemiological study indicates that green tea consumption prevents type-II diabetes effectively (Ryu and others 2006). Green tea extracts can modify glucose metabolism

both in animal models (Henning and others 2015; Sundaram and others 2013) and human trials (Li and others 2015; Toolsee and others 2013; Venables and others 2008). Unlike green tea, black tea could help generate β cells in pancreas (Tang and others 2013). Berries are phenolic-rich fruits, and it has been noted for their outstanding health effects, partly due to high phenolic content and antioxidant activity (Johnson and others 2011). Growing evidence indicates that berries especially blueberry extracts are effective for decreasing blood glucose in animal models (Grace and others 2009). Martineau tested the anti-diabetic activity of ethanol extracts from roots, leaves and fruits of wild blueberry *in vitro* and found extracts significantly increased glucose transport in C2C12 muscle cells (Martineau and others 2006).

In addition, it has been found that vegetables such as kale, beets, broccoli, spinach, potato, carrots and cabbage had high antioxidant activities (Cao and others 1996). Recently, some studies showed that the phenolic compounds obtained from vegetables could reduce the risk of developing obesity and other metabolic diseases (Donado-Pestana and others 2015).

Beside the foods mentioned above, cherry, grape, lemon, spinach, broccoli, red cabbage, green chili pepper, black bean, black soybean, turtle bean, grape juice and red wine are also phenolic-rich foods (Amarowicz and others 2008). However, there are hundreds of genotypes of legumes, and phenolic substances in different genotypes are significantly different (Xu and Chang 2007).

2.3 An overview of legumes

2.3.1 Legume consumption in the United States

Legumes and their products play a vital role in traditional diets in many regions not only in Asia but also in Europe and America (Geil and Anderson 1994). Tofu (Cai and Chang 1999), soy sprouts (Megat Rusydi and Azrina 2012), tempeh (Astuti and others 2000) and soymilk (Liu and Chang 2013) are consumed by people all over the world. In the United States, during the time periods of 1909-1913, 1967-1969, and 1985, the consumption of dry beans, soybeans and peas combined remained constant at 7.3 Kg, 7.3 Kg, and 8.2 Kg per person per year, respectively (Messina 1999). The dry edible bean consumptions in years 1972, 1981, 1982 and 1992 were 2.7Kg, 2.5 kg , 3.0 kg, and 3.4 kg (Messina 1999) and for years of 2011, 2012 and 2013, dry beans, peas and lentil consumptions were 2.95 kg, 3.13 kg and 3.22 kg per person per year, respectively (Thornsbury and others 2013). In the US Department of Agriculture food guide pyramid, legumes are in the same group as meat, poultry and fish (Willett and others 1995). The major beans people consumed in the United States were navy bean, black bean, pinto bean, kidney bean and lima bean, during the time period of 1997-1999, the consumption of black bean, pinto bean, navy bean and kidney bean were 0.22 kg, 1.63 kg, 0.57 kg and 0.27 kg, respectively (Lucier and others 2000). However, legume consumption seems to have a poor future in contrast to the amazing nutritional value it offers.

2.3.2 Nutrient composition in legume

2.3.2.1 Protein

The range of protein content in edible legumes is from 20% to 30%, generally. The quality of legume protein is often underestimated, even though legumes are

recognized as high protein food. Protein-efficiency ratio (PER) is mainly responsible for the underestimation. Until recently, the growth of laboratory rats was the standard method for testing protein quality. However, the methionine requirement for rats is 50% higher than human beings. Legume proteins are low in the sulfur amino acids. As a result, the protein efficiency ratio is much lower than the real value (Sarwar and others 1984).

However, in terms of calcium retention, the low sulfur amino acid may offer an advantage. Metabolism of sulfur amino acid is partly responsible for the hypercalciuric effect of protein. Hydrogen ions produced from the metabolism of sulfur amino acids lead to the demineralization of bone to produce calcium ions in the urine (Remer and Manz 1994). Thus, calcium retention might be improved by consuming legumes. Some studies showed that legume consumption is related to a distinctly lower urinary calcium excretion, especially compared with the consumption of whey protein (Marckmann and others 2015) or a mixture of animal proteins (Curhan and others 1997). In general, legume protein only provides a very small portion of total dietary protein intake even among people who are vegetarian (Messina 1999).

However, Food and Drug Administration approved the health claim that 25 g/day of soy protein, as part of a low saturated fat and cholesterol will decrease the risk of developing cardiovascular diseases in 1999 (FDA 1999), meanwhile, a human study indicated that substituting soy protein for animal protein decreases the level of total cholesterol, LDL cholesterol and triglyceride significantly (Crouse and others 1999).

2.3.2.2 Fat

Generally, fat content in food legume is less than 5%. However, chickpea and soybean contain around 8% (Jukanti and others 2012) and 20% fat (Redondo-Cuenca

and others 2007), respectively. Linoleic acid is the major fatty acid consumed in legumes, although legumes contain other fatty acids, including n-3 fatty acid such as α-linolenic acid (Hepburn and others 1986) and stearidonic acid (Chen and others 2006). However, total fat content in common beans is quite low, and therefore the fatty acids from common edible bean have minor contribution to the whole dietary fatty acid consumption. For the soybean and its related products, which have higher fat content than other legumes, the intake of α-linolenic acid is significantly higher (Messina 1999).

2.3.2.3 Fiber and glycemix index

Legumes are good sources of dietary fiber (Mojica and others 2015). High fiber diet has been shown to decrease the serum cholesterol in hypercholesterolemic subjects (Brown and others 1999), lowers postprandial plasma glucose concentrations of type-II diabetes (Chandalia and others 2000), lowers the incidence of coronary heart diseases (Rimm and others 1996), reduces blood pressure of hypertension individuals (Whelton and others 2005), and brings down the risk for obesity (Liu and others 2003), and it is also inversely related to total cancer death (Pierce and others 2007).

In terms of glycemic index, legumes have shown low glycemic index (Atkinson and others 2008). Researchers published thousands of paper during the past decades even though neither American Diabetes Association nor the American Dietetic Association supports the glycemic index as a reference for individuals with diabetes. As a matter of fact, glycemic index of foods is one important factors affecting the overall quality of people's diet. In support of this statement are the findings from an impressive study showing that high dietary glycemic index is associated with increased risk of type-II

diabetes (Bhupathiraju and others 2014). Therefore, legumes may be an important food for people with diabetes and those with a high risk of developing diabetes.

2.3.2.4 Legume phenolic compounds and their antioxidant determination methods

In recent years, colored common beans such as black bean (*Phaseolus vulgaris L.*), black soybean (*Glycine max L.*), lentil (*Lens culinaris*) have attracted increasing attention because of their phenolic compounds and their health-promoting effects which are related to prevention of chronic diseases.

To study phenolic composition and antioxidant activity in legumes, our group has carried out a comprehensive study to optimize the yield by selected solvent systems (Xu and Chang 2007). In this study, six solvent systems with different polarities were used for the extraction of phenolic substances from eight classes of legumes. Three different antioxidant assays (FRAP: ferric-reducing antioxidant power; DPPH-radical scavenging ability; and ORAC: oxygen radical absorbance capacity) with different mechanisms (Dudonne and others 2009; Prior and others 2005) were used to determine the antioxidant properties. The results showed that different legumes required different solvent systems for maximizing the yield of different phenolic substances. Furthermore, we have studied phenolic substances, antioxidant capacity and the surface color of thirty-three legume cultivars (Xu and others 2007). The results indicated antioxidant capacities of legumes were strongly correlated with total phenolic content, but only weakly associated with the surface color. Therefore, not all colored legumes have high antioxidant capacities. As reported in our earlier study, black soybean, black turtle bean and lentil have the highest total phenolic content and antioxidant capacities (Xu and others 2007). Oomah and

coworkers found black bean had the highest level of flavonol and anthocyanin, and the major contributor is seed coat pigment, when compared with three other soybeans which had colored hull (Oomah and others 2010).

Phenolic compounds have shown to have colorectal cancer-prevention (Zhu and others 2015), breast cancer-prevention (Shu and others 2001), cardiovascular disease prevention (Kris-Etherton and others 2002), α-amylase inhibition (Apostolidis and Shetty 2008; Links and others 2015), α-glucosidase inhibition (Zhang and others 2015; Zhang and others 2011), lipase inhibition (Calabrone and others 2015; Zhang and others 2015), and angiotensin-converting enzyme inhibition (Connolly and others 2015) ability and hypocholesterolaemic effects (Ferreira and others 2015). Salicylic acid, syringic acid, 2,3,4-trihydroxybenzoic acid, sinapic acid, caffeic acid, gallic acid, vanillic acid, chlorogenic acid, myricetin, (+)-catechin, (+)-epicatechin gallate, delphinidin-3-glucose, malvidin-3,5-diglucose, petunidin-3-glucose, malvidin-3-glucose are the major phenolic compounds in black bean and black soybean (Xu and Chang 2008, 2009).

It should be noted that a single antioxidant determination method might function through multiple mechanisms or through only one mechanism dependent on the system in which it exerts action. In addition, antioxidants respond differently to different radicals or oxidation sources (Prior and others 2005). Hence, a combination of multiple assays is a better choice to obtain a more comprehensive antioxidant profile (Beretta and others 2005).

2.3.2.5 Antioxidant capacity and enzyme inhibition

Some studies indicated that antioxidant capacity are related to enzymes (linked to diabetes and hypertension) inhibition ability (Apostolidis and Lee 2010; Raphael and

others 2002; Shibano and others 2008). Mcdougall and coworkers reported that α-amylase and α-glucosidase inhibition abilities were highly related to different phenolic components. However, they have not determined correlations between antioxidant activity and enzymes inhibitory ability (McDougall and others 2005). Meanwhile, it has been observed phenolic extract of Oolong tea had lipase inhibitory ability, and the IC_{50} of epigallocatechin gallate (EGCG) against lipase was 0.349 mM (Nakai and others 2005), EGCG isolated from tea was also reported had high antioxidant activity (Hashimoto and others 2003). It should be pointed out that antioxidant activity is not the only indicator for enzymes inhibition ability, other parameters such as specific structures and specific phenolic substance contents also may play important roles.

2.3.2.6 Non-nutritive components

Legumes contain some components thought to be anti-nutrients in the past, such as trypsin inhibitor, phytate and saponins. Trypsin inhibitors from legumes can interfere with protein digestion, and will lead to pancreatic enlargement in some species of animals (Friedman and Brandon 2001). However, boiling dry bean is an effective way to inactivate trypsin inhibitor by 80% to 90% (Rayas-Duarte and others 1992). In contrast to trypsin inhibitor, chymotrypsin inhibitor (Bowman-Birk inhibitor) found in legumes has anti-cancer effects (Kennedy and Wan 2002). Phytate in legumes was thought to have the ability to decrease mineral bioactivity; however, phytic acid may reduce the risk of colon cancer due to its antioxidant activity (Lai and others 2013). For saponins, it has been reported that saponins can inhibit the proliferation of tumor cells (Gao and others 2013) and had high suppressive effect on the growth of Caco-2 and HT-29 cells (Lai and others 2013).

2.4 Plant phenolic compounds extracts

2.4.1 Parameters for phenolic extraction

Generally, people use either organic or inorganic solvents to extract plant phenolic compounds. Several factors may influence the yield of phenolics, including extraction time, temperature, solvent-to-sample ratio, as well as solvent type. In addition, the recovery of phenolics varies from sample to sample and also depends on the type of plants and their active compounds. Different solvent systems have been used to get the phenolic compounds in plants such as berries, nuts, vegetables, legumes and other food stuffs (Sun and Ho 2005). For extracting crude phenolic compounds from plants, 80% acetone aqueous (Ademiluyi and Oboh 2013), 70% ethanol aqueous (Ismail and others 2004; Kim and others 2011), absolute ethanol (Da Porto and others 2013), 50% ethanol aqueous (Gawlik-Dziki 2008), and solvent mixtures (methanol : acetic acid : water = 25 : 1 :24) (Papandreou and others 2009) have been used. The differences in extraction efficiencies could be partly due to the character of phenolic compounds in different types of plant. In choosing extraction solvent, there are two major factors that affect the yield significantly: time and temperature. Theoretically, increasing extraction time and temperature can accelerate phenolic dissolving in solvent. However, phenolics can be degraded or oxidized with the increase in time and temperature (Biesaga and Pyrzyńska 2013; Davidov-Pardo and others 2011). The volume/weight ratio of solvent-to-sample also affects the recovery of phenolics. There is no doubt that increasing the ratio of solvent to sample will promote the phenolic yield. However, in terms of economy, determining the best ratio is necessary so that solvent input is minimized and the yield is maximized. A 60 : 1 (V/W) ratio is enough for most phenolic extractions (Al-Farsi* and

Lee 2008). Particle size and sample matrix also significantly influence the phenolic yield, because some phenolics can bind to other components such as proteins and carbohydrates, and these associations can be broken down by adding some enzymes to accelerate the phenolic release from materials (Pinelo and others 2008).

2.4.2 Techniques for phenolic extraction

2.4.2.1 Microwave-assisted method

Apart from a single extraction method just using organic solvent, some other modified methods have been developed. The advantages of a microwave-assisted method as compared to single solvent extraction include minimized usage of solvent, shortened extraction time and increased extraction yield (Huie 2002). In terms of mechanism, microwave can make solvents containing polar molecular vibrate in materials to produce heat (Camel 2001). Heating can take moisture of cell away through evaporation, and steaming can break cells to let them release the active compounds (Wang and Weller 2006). Microwave-assisted methods can be applied to phenolic extraction, since phenolics are dipoles and the hydroxyl groups inside can absorb microwave energy (Ajila and others 2011). Since microwave-assisted method is influenced by many parameters, some statistical studies have been done to determine the best processing conditions to extract different phenolic compounds (Proestos and Komaitis 2008; Vasu and others 2010).

2.4.2.2 Sonication-assisted method

Sonication-assisted method, which uses frequencies of ultrasonic radiation higher than 20 KHz accelerates releasing phenolic compounds from materials into solvents.

Sonication can break cell walls through cavitation bubbles produced from sound waves, and therefore the cell contents can release faster (Vinatoru 2001). There are many parameters influencing extraction recovery beside sonication time, temperature and solvent, such as sonication frequency and ultrasonic wave distribution (Wang and Weller 2006). People also used sonication-assisted methods to extract phenolic and anthocyanin from jabuticaba peel (Rodrigues and others 2015) and flaxseeds (Corbin and others 2015). Roidaki compared the antioxidant activity and total phenolic contents in extracts, which were extracted with a sonication-assisted method and conventional solvent method, and results showed that the sonication-assisted extracts had higher antioxidant activity and total phenolic contents (Roidaki and others 2015). Compared with microwave-assisted method, sonication-assisted methods are much cheaper and simpler, which may be operated easily and widely in the industry (Lee and Lin 2007).

2.4.2.3 Hydrolysis-assisted method

As mentioned above, phenolics can bind to other cellular components such as proteins and carbohydrates (Pinelo and others 2008), and some cell wall of plant tissue are very thick, which can prevent cell contents from releasing into solvents (Li and others 2006). The thick cell walls and the linkage between phenolics and proteins or carbohydrates can be hydrolyzed by adding enzymes. However, some proteins can be co-extracted with the addition of enzymes (Li and others 2006; Pinelo and others 2008) such as protease (Landbo and Meyer 2001). Apart from adding enzymes, alkalis and acids have also been applied in phenolic extraction. Kim and coworkers use the alkaline conditions to separate bonded phenolics from wheat bran (Kim and others 2006).

2.4.3 Purification of phenolic compounds by adsorption

2.4.3.1 Activated carbon adsorbent

Small hydrophobic graphite layers are the main ingredients of activated carbons with disordered, irregular and heterogeneous surface. The adsorbent properties of activated carbons come from their composition, physicochemical properties and mechanical strength (Marsh and Reinoso 2006). Surface modification, induced by physical activation and by chemical activation or by a combination of both have been considered in order to control the pore size and porosity (Ioannidou and Zabaniotou 2007; Jones and others 2002; Marsh and Reinoso 2006; Menéndez and others 2010; Yin and others 2007). Activated carbons produced from low-cost materials such as coal and agricultural by-products are good sources of commercial activated carbons. Lots of agricultural waste are potential raw materials for producing commercial activated carbons, such as jackfruit peel (Jain and Jayaram 2007), waste from cherries (Shopova and others 1997), fruit shell, seed coat and husk (Evans and others 1999; Galiatsatou and others 2002; Tan and others 2008).

2.4.3.2 Mineral adsorbent

Mineral adsorbents include clay, natural zeolites and siliceous materials. Chemical modification can increase the affinity of minerals (Huang and others 2008). Adsorption of phenolics from olive mill wastewater and krafe mill effluents with sepiolite have been studied (Ugurlu and Hazirbulan 2007). Study of mineral adsorbent is rare because mineral adsorbent making is a complex process that involves different mechanisms (Chen and Wang 2007).

2.4.3.3 Resin adsorbent

Resin is a synthetic polymer made of materials with hydrophilic and hydrophobic characters. Polymeric adsorbents are long lasting, stable and possess high adsorption ability. Also, they are easy to regenerate, though the effective surface area is smaller than of activated carbons. Ion-exchange consists of polymer matrixs, polysaccharides, synthetic resins, functional groups and inorganic compounds. Depending on the positive and negative charges of ion-active groups, resin acts as cation or anion exchangers according to surface charges. Resin column chromatography is a commonly used method to purify crude phenolic compounds. AB-8 resin-based column chromatography has been used for the purification of four kinds of flavones (Zhang and others 2008). Ionic XAD4, XAD16, and XAD-7 resins can be used for the recovery of phenolics and their separation from carbohydrates (Zagklis and others 2015). Resin X-5, D-101, H-103, S-8, NKA-9 and AB-8 have been used to remove salt and impurities in crude phenolic compounds (Feng and others 2015), and XAD-7 resin is used to remove sugar and impurities from crude phenolic extracts of legumes (Zou and others 2011).

2.4.3.4 Biosorbent

The most commonly used biosorbent is obtained from the wastes of fermentation and activated sludges. This kind of biosorbent not only has a wide source, but also has a low-cost. Sewage sludge may be a potential low-price biosorbent for phenolic purification (Smith and others 2009). However, toxicity of biosorbent needs be considered.

2.4.3.5 Polysaccharide-based adsorbents

Polysaccharide-based adsorbents include chitosan, chitosan–cyclodextrin derivatives, Sephadex, starch, cross-linked starch, starch derivatives, and hybrid materials (Delval and others 2006; Li and Chase 2009; Romo and others 2008). Polysaccharide-based adsorbents show high stability, reactivity, adaptability and selectivity. Some functional groups show great chelation properties enabling ease of regeneration (Soto and others 2011). It is well known that polysaccharide-based adsorbents are renewable and have an ability to interact with a variety of molecules through physical or chemical interactions (Crini 2005). Troszynska and coworkers observed fractions coming from Sephadex LH-20 chromatography with high phenolic content (Troszynska and others 1997). Phenol, p-nitrophenol and p-chlorophenol are removed from aqueous solution by using cross-linked β-cyclodextrin polymer (Li and others 2009), and phenols can be removed from aqueous medium by using chitosan-calcium alginate (Nadavala and others 2009).

2.5 An overview of type-II diabetes

2.5.1 Prevalence of type-II diabetes

Type-II diabetes is first reported as a metabolic disease (Patlak 2002), and it is also called non-insulin dependent diabetes mellitus, which is the most common form of diabetes mellitus characterized by hyperglycemia, insulin secretion and action disorder (Withers and others 1998). The prevalence has been increasing rapidly all over the world. About 366 million people suffered from diabetes worldwide in 2011; however, the number will be doubled by 2030 (Ginter and Simko 2013). Meanwhile, the number of people diagnosed with diabetes is increasing in every country, with 80% of diabetes

patients living in low or middle income countries (Olokoba and others 2012). In 2011, 4.6 million people died from diabetes and its complications in the United states (Abegunde and others 2007). In the of United States, diabetes affected about 8% population in 2010 and 90% to 95% of the patients were diagnosed with type-II diabetes (Control and others 2011). However, no cure has been found for the type-II diabetes even though numerous people are suffering from it all over the world.

2.5.2 Life style, genetics factors

Even though no cure has been found yet, we still have some ways to protect people from type-II diabetes and relieve pain, such as life style modifications (Ramachandran and others 2006), oral hypoglycemia drugs and sensitizers like metformin (Group 2002). As we all know, type-II diabetes is mainly due to life style and genetics (Ripsin and others 2009). Second, life style is the key parameter for developing type-II diabetes, such as sedentary life style (Healy and others 2008), lacking physical exercises (Booth and others 2008), and high fat or high carbohydrate diet (Hu and others 2001).

There is a high inheritable connection in type-II diabetes, which means having an immediate family member with type-II diabetes significantly increases the risk of developing type-II diabetes (Olokoba and others 2012). TCF7L2, PPARG, FTO, KCNJ11, NOTCH2, WFS1, CDKAL1, IGF2BP2, SLC30A8, JAZF1, and HHEX. KCNJ11 genes are reported to be significantly related with type-II diabetes. For example, TCF7L2 regulates the gene expression of proglucagon and the production of glucagon-like peptide; therefore TCF7L2 is an important gene for type-II diabetes (Da Silva Xavier and others 2012).

2.5.3 Enzymes linked to type II diabetes

Current remedial options for metabolic syndromes involve lifestyle modification and poly-pharmacological treatments. However, improved therapeutic and preventive approaches are needed (Cherniack 2011). Controlling postprandial hyperglycemia is one of the approaches to manage diabetes (Ceriello 2005), and this can be achieved by having low glycemic index diet (Rizkalla and others 2004) or inhibiting enzymes, which can hydrolyze carbohydrates (Ortiz-Andrade and others 2007) and lipids (Pilichiewicz and others 2003). In terms of carbohydrate, the key enzymes are α-amylase, α-glucosidase and maltase (Bhandari and others 2008). Starch or glycogen can be hydrolyzed by α-amylase into glucose and maltose, and α-glucosidase hydrolyzes terminal non-reducing 1-4 linked alpha-glucose residues to release a single alpha-glucose molecule; maltase catalyzes the hydrolysis of maltose to single glucoses (Worthington 1988). Figure 2.4 shows the cleavage points of enzymes in the digestion of starch. There are hundreds of published studies, indicating that phenolic extracts have inhibitory activity against α-amylase and α-glucosidase. Phenolic extract from finger millet shows high inhibition rate for both α-amylase and α-glucosidase (Shobana and others 2009); phenolic compounds from traditional herb plant in Latin American possessed a high antioxidant activity and inhibitory activity against both angiotensin I-converting enzyme and α-amylase (Ranilla and others 2010). High inhibition ability of α-glucosidase produced by the phenolic extracts from 20 Canadian lentil cultivars has been observed (Zhang and others 2015). However, not all phenolic extracts show significant inhibition against both α-amylase and α-glucosidase. For example, the phenolic extract from grape skin only shows significant inhibition against α-glucosidase but not α-amylase (Zhang and others 2011).

In terms of lipids, the major lipid present in dietary sources is triacylglycerol (Svendsen 2000). Lipase plays a very important role in digestion, transportation and processing of dietary lipids. This enzyme is the main enzyme hydrolyzing triglyceride to a monoglyceride and two fatty acids (Worthington 1988). Figure 2.5 shows lipid digestion and absorption. Lipid digestion is not directly linked to diabetes. However, diabetes is highly prevalent in obese population. Therefore, lipase activity is included in this study. Recently, not only the inhibition of α-glucosidase by phenolic extracts was observed, the inhibition of lipase was also reported. As mentioned above, the phenolic extracts from 20 Canadian lentil cultivars also show high inhibition rate against lipase (Zhang and others 2015) and the strawberry phenolic extracts show inhibitory ability against lipase *in vitro* (McDougall and others 2009). In animal study, the extract of *Nelumbo nucifera* leaves has been reported to have inhibitory activity of lipase and α-amylase, and it can up-regulate lipid metabolism (Ono and others 2006). However, not all studies show positive results, among which the phenolic extract from grape skin has no significant inhibition against lipase (Zhang and others 2011).

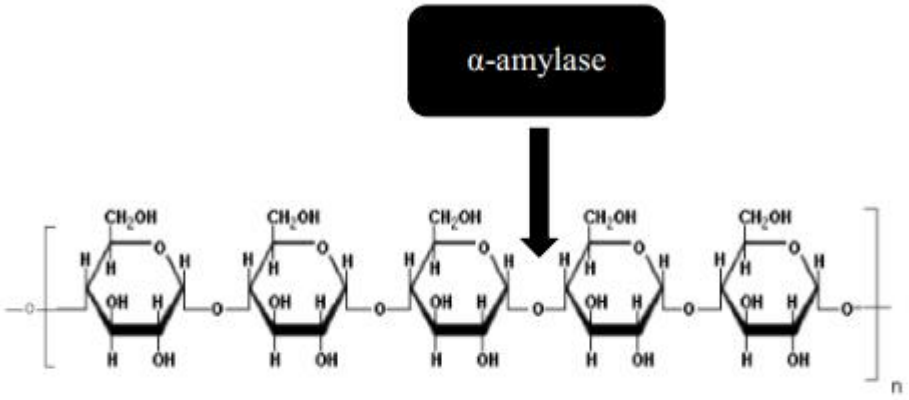


Figure 2.4 Cleavage points of enzymes in the digestion of starch

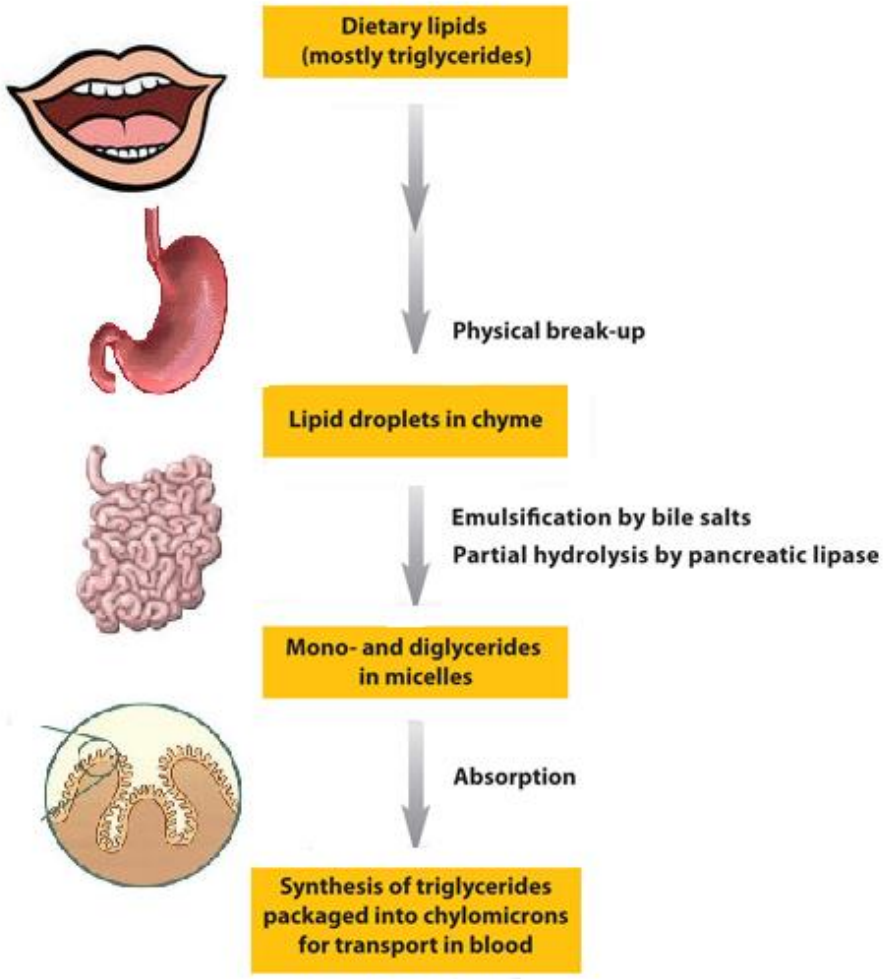


Figure 2.5 Lipid digestion and absorption

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Legumes, berries, tea, broccoli and red cabbage

All food materials except for tea and legumes were obtained from local supermarket, green tea and black tea were ordered from Teavana (Teavana, GA, US), black bean and black soybean were from Goya (Secaucus, NJ, US) and all materials were stored at -20^{o}C until use.

3.1.2 Chemicals

(+)-Catechin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein disodium salt, Folin-Ciocalteu reagent, gallic acid (GA), sodium carbonate, aluminum chloride, sodium nitrite, sodium hydroxide, condensed hydrochloric acid, vanillin, 6-hydroxy-2,5,7,8-tetramethlchroman- 2-carboxylic acid (Trolox), potassium phosphate dibasic, sodium phosphate monobasic, 2, 2´-azobis (2-amidinopropane) dihydrochloride (AAPH), sodium phosphate dibasic, sodium chloride, sodium potassium tartrate, isopropanol, 3,5-dinitrosalicylic acid, potato starch, sodium cholate, gum arabic from acacia tree, lipase from porcine pancreas (EC 3.1.1.3), α-amylase from porcine pancreas (EC 3.2.1.1), α-glucosidase (EC 3.2.1.20), p-nitrophenyl-β-D-glucopyranoside (pNPG), voglibose, dimethylsulfoxide, 4-nitrophenyl palmitate (PNPP), α-amylase inhibitor from *Triticum aestivum* (wheat seed), lipase inhibitor, Amberlite® XAD-7, Sephadex® LH-20,

methanol, ethanol and acetone were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A).

3.2 Methods

3.2.1 Crude phenolic extraction

According to our previous study (Xu and Chang 2007), beans were finely ground with a Retsch ZM 200 ultra centrifugal mill (Retsch GmnH, Germany). One gram of bean powder was extracted in a 15 mL centrifuge tube with 10 mL solution including acetone/acetic acid/ water (70/0.5/29.5, v/v/v), and the mixture was shaken at 150 rpm in a VWR standard analog shaker (West Chester, PA., U.S.A.) at room temperature for 3 h. After 3 h, the mixture was centrifuged by Thermo Legend X1R centrifuge (Thermo Scientific Inc. Waltham, MA, U.S.A.) at 1200 × g for 15 min. The supernatant was transfered to another tube and the residue was re-extracted for 12 h with 10 mL extraction solution. After centrifugation, two supernatants were combined. Organic solvent in the extracts were removed by a rotary evaporator (BÜCHI Labortechnik AG, Switzerland) under vacuum at 38^{o}C. The crude black bean and black soybean extracts were obtained by lyophilizing the concentrated extracts and stored at -20^{o}C until use.

Crude extracts were obtained by the method reported previously with some modifications (Velioglu and others 1998). Berries were ground with a blender (Oster Co. Milalkee, WI, US) at high speed for 3 min, and then freeze-dried. Ten grams of the freeze-dried powder were extracted in a conical flask with 100 mL extraction solution (70 % ethanol). The mixture was shaken at 150 rpm in a VWR standard analog shaker (West Chester, PA., U.S.A.) at room temperature for 3 h, then the mixture was filtered through Whatman No.4 filter paper to remove residue. The residue was re-extracted with 50 mL extract

solution for an additional 12 h in the dark; and the two extract solutions were combined. Organic solvent was removed using a rotary evaporator under vacuum at 38ºC. The concentrated extract was lyophilized and designated as crude extract, and stored at -20ºC until use.

Extraction method was performed according to Pan and coworkers with a slight modification (Pan and others 2003). Ten grams of black tea or green tea were mixed with 100 mL 50% ethanol. The suspensions were irradiated with 900 W microwaves (Rival Co. Kansas, MO., U.S.A) as follows: 45 s power on (heating to the desired temperature about 85-90 ºC), 10 s power off and then 3 s power on (for heating) and 10 s power off (for cooling). This cycle was repeated four times. Super-boiling of the solution was prevented from taking place. After extraction, the mixture was filtered through Whatman No.4 filter paper to remove residue, then organic solvent was removed using a rotary evaporator under vacuum at 38ºC. The concentrated extract was lyophilized and stored at -20ºC until use.

Extraction method was done according to Ismail and coworkers with some modifications (Ismail and others 2004). One kilogram of fresh broccoli or red cabbage was cleaned and washed with tap water, and excessive water was dripped off and air-dried by a fan. One hundred grams of edible sample were cut into small pieces and homogenized using a wet blender for 3 min. The homogenized sample was freeze dried and kept at -20ºC until use. Ten grams of freeze-dried sample were mixed with 70% ethanol and stirred at 150 rpm for 1 h at the room temperature, and the extract was filtered through Whatman No.4 filter paper to remove residue, which was re-extracted twice, then the three extracts were combined. The organic solvent in the extract solution

was removed using a rotary evaporator under vacuum at 38ºC. The concentrated extract was lyophilized and stored at -20ºC until use.

3.2.2 Removal of sugars from crude extracts

Sugar removal was performed by column chromatography column which was packed with Amberlite® XAD-7 resin. According to Hung and Yen (2002) with some modifications, four grams of crude extract were dissolved in 20 mL of distilled water by vortexing vigorously. The mixture was 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 μm membrane to obtain a clear solution. The clear solution was gently poured into the column (column of 50 × 2.6 cm, i.d., bed volume (BV) = 180 mL) and eluted with distilled water at a speed of 1.5 BV/h.

The resin was washed with 2 BV of distilled water to remove sugars, organic acid and other water soluble components. Then 80% methanol was used to elute the phenolic compounds at the speed of 3 BV/h to collect the phenolic fraction. Methanol in the effluent was removed using a rotary evaporator under vacuum at 38 ºC, and the concentrated was freeze-dried to produce dried powder, which was designated as semi-purified extracts, which were stored at -20ºC until use.

3.2.3 Fractionation of semi-purified extracts

Fractionation of semi-purified extracts was carried out as reported by Zou and coworkers (Zou and others 2011). One hundred milligrams of semi-purified extract were suspended in 1mL distilled water and vortexed vigorously. The sample was then filtered through a 2 μm membrane to remove insoluble residue. The pre-treated sample was

loaded to the column (100 × 1.6 cm, i.d., BV= 200 mL) packed with Sephadex LH-20. The column was eluted with distilled water (600 mL), 50% ethanol (600 mL), and 50% acetone (600 mL) sequentially at a flow rate of 0.5 mL/min. The elution was monitored at 280 nm by an UV detector. Figure 3.1 shows the flow chart of fractionation of black soybean extract. Figure 3.2 shows the flow chart of fractionation of black bean extract.

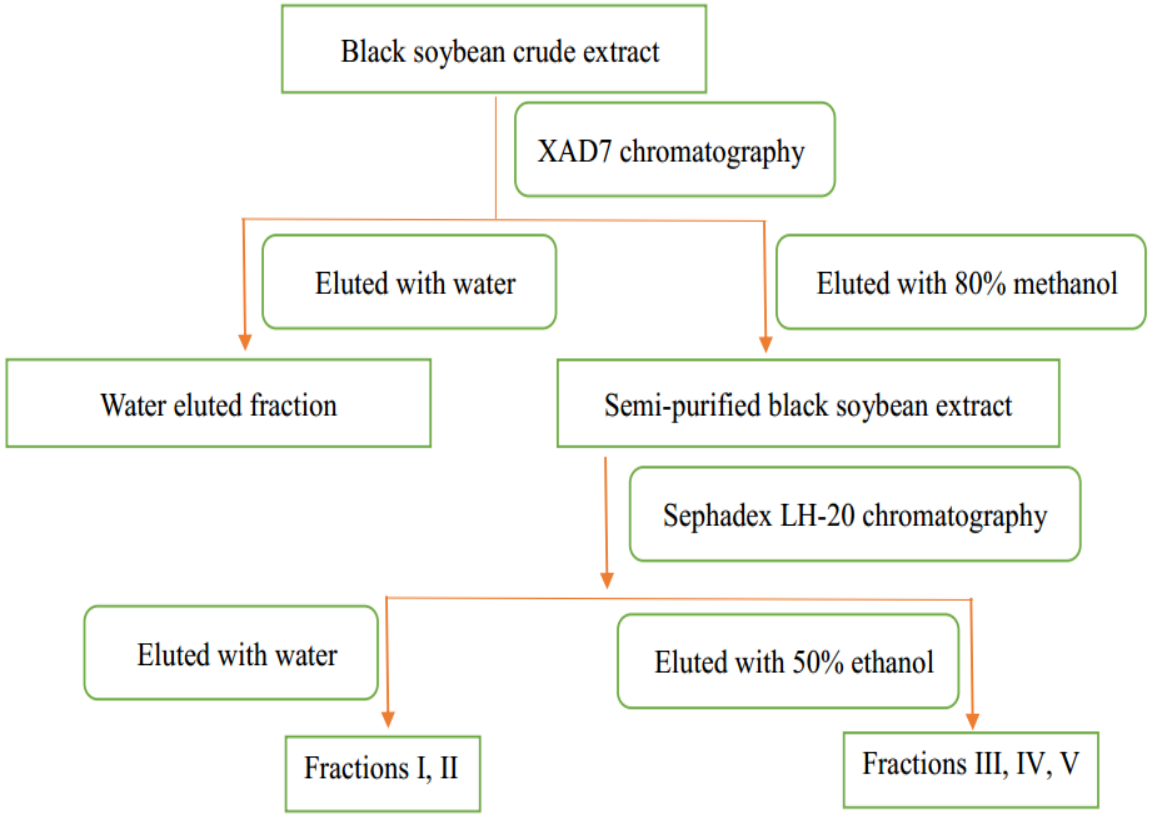


Figure 3.1 Flow chart for the fractionations of black soybean phenolics.

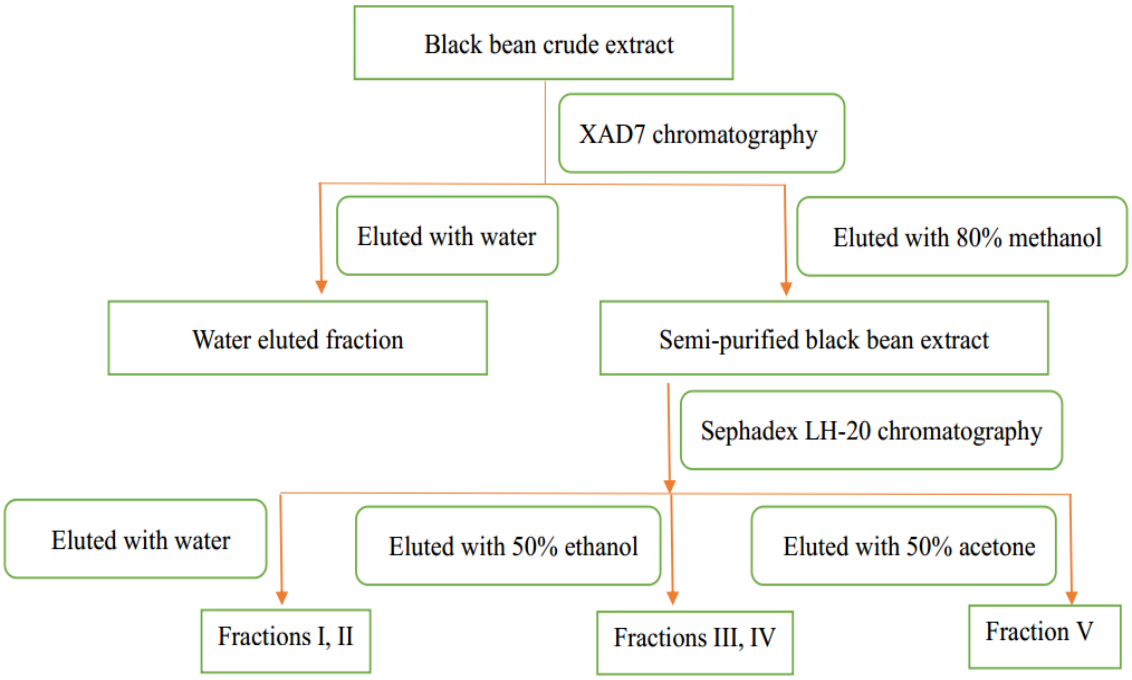


Figure 3.2 Flow chart for the fractionation of black bean phenlics.

3.2.4 Total phenolic content (TPC) determination

Total phenolic content (TPC) was determined by a Folin-Ciocalteu assay with some modifications (Xu and Chang 2007). In brief, the mixture of 10 µL of the sample solution with appropriate dilution, 0.6 mL distilled water, 50 µL Folin-Ciocalteu's reagent solution, and 150 µL 7% Na_2CO_3 were added into a 1.5 mL centrifuge tube. The mixture was vortexed to mix well and incubated for 8 min at room temperature. Then 190 µL distilled water were added into the tube. The final mixture was allowed to stand for 2 h at room temperature. Two-hundred microliters of mixture were taken from the tube and added into wells of 96-well plate, the absorbance was measured with a plate reader (Molecular Devices, CA, U.S.A.) at 765 nm against gallic acid standard. Total phenolic

content was expressed as gallic acid equivalents per gram of freeze-dried sample (mg of gallic acid equivalent/g food).

3.2.5 Total flavonoid content (TFC) determination

Total flavonoid content was determined according to previous method (Xu and Chang 2007). Briefly, 50 µL sample with appropriate dilution were mixed with 250 µL distilled water in 1.5 mL centrifuge tube. Then 15 µL of 5% $NaNO_2$ were added, mixed and allowed to stand for 6 min. Thirty microliters of 10% $AlCl_3 \cdot 6H_2O$ were added, another 5 min later, 100 µL 1 N NaOH and 45 µL distilled water were added and mixed well. Two-hundred microliters were added into wells of 96-well plate, the absorbance was measured with a plate reader (Molecular Devices, CA, U.S.A) at 510 nm against (+)-catechin standard. The results were expressed as (+)-catechin equivalents per gram of freeze-dried sample (mg of catechin equivalent/g food).

3.2.6 Condensed tannin content (CTC) determination

Condensed tannin content was determined by previous method with a slight modification in our laboratory (Xu and Chang 2007). In brief, 10 µL sample with appropriate dilution were mixed with 0.6 mL 4% methanol vanillin solution and 0.3mL concentrated hydrochloric acid in 1.5 mL centrifuge tube, and the mixture was allowed to stand for 15 min. Two-hundred microliters of the mixture were added into wells of 96-well plate, and the absorbance was measured with a plate reader (Molecular Devices, CA, U.S.A) at 500 nm against (+)-catechin as standard. The content of condensed tannins was expressed as (+)-catechin equivalents per gram of freeze-dried food sample (mg of catechin equivalent/g food).

3.2.7 Analysis of radical DPPH scavenging activity

DPPH-free scavenging capacity of legume extracts was determined according to the our previously study (Xu and Chang 2007). Briefly, 20 μL of extract or fractions with appropriate dilution were mixed with 380 μL of 0.1mM DPPH solution, mixed well by vortexing and allowed to stand at room temperature in the dark for 30 min. Two hundred microliters of the mixture were taken and added into wells of 96-well plate, the absorbance of the sample (A_{sample}) was measured at 517 nm against an ethanol blank, a negative control ($A_{control}$) containing 3.8 mL DPPH solution and 0.2 mL of the 100% ethanol. The percentage of DPPH discoloration (free radical scavenging rate) of the sample was calculated according to the following equation:

$$\text{Percentage discoloration} = [1 - (A_{sample}/A_{control})] \times 100 \quad (3.1)$$

The free radical scavenging activity of sample was expressed as micromoles of Trolox equivelents per gram (μmol trolox equivalent/g) of freeze-dried extracts or fractions.

3.2.8 Oxygen radical absorbing capacity (ORAC) assay

The ORAC assay was conducted as reported with slight modifications (Xu and Chang 2007). A plate reader (Molecular Devices, CA, U.S.A) equipped with adjustable fluorescence filters and incubator was used, and the temperature of incubator was set at 37 °C, excitation wavelength of fluorescence filter was set at 485 nm and emission wavelength was set at 520 nm. Kinetic reading was recorded for 60 cycles of 40 s each. AAPH was used as free radical initiator, Trolox was used as the standard, and all of them were dissolved in phosphate buffer (75 mM, pH 7.0), phosphate buffer was used as blank. The samples were diluted with phosphate buffer (75 mM, pH 7.0) to the appropriate

concentration to fall within the linearity range of the standard curve. After adding 20 µL of sample, standard and blank, and 200 µL pre-headed fluorescein solution were added into appointed wells. After the 96-well plate was incubated in a plate reader for 30 min, 20 sµL of AAPH solution (3.2 µM) was added to activate the reaction. Kinetics of the fluorescence changes were recorded immediately by software SoftMax Pro (Molecular Devices, Sunnyvale, CA, U.S.A). The ORAC value was calculated and expressed as micromoles of Trolox equivalent per gram sample (µmol of trolox equivalent/g) using the standard curve of Trolox.

3.2.9 α-Amylase inhibition assay

α-Amylase inhibitory activity was determined per earlier reported method with slightly modification (Zhang and others 2011). Forty microliters of legume extract or individual purified phenolic compounds, 160 µL of distilled water and 400 µL 0.5% starch were mixed in 1.5 mL centrifuge tube. After adding of 200 µL of the enzyme solution (30 unit/ mL), the tubes were incubated at 25ºC for 3 min. Then, 200 µL mixture were removed and added into a separate tube, which contained 100 µL DNS color reagent solution (96 mM 3,5-dinitrosalicylic acid, 5.31 M sodium potassium tartrate in 2 M NaOH). The tubes were placed into a 95 ºC thermo mixer (Eppendorf, Hamburg, Germany) for 10 min to inactivate the enzyme. Nine-hundred microliters of distilled water were added into each tube and mixed well. Then 200 µL of mixture were taken and added into wells of 96-well plate. The absorbance of the samples was measured at 540 nm. To eliminate the absorbance produced by legume extracts itself, appropriate extract control without enzymes was included. The percentage of inhibition was calculated by

following equation. The inhibition rate was determined at five different concentrations of legume extract to get IC_{50} (mg/mL).

$$\alpha\text{-Amylase inhibition\%} = [(A_{sample} - A_{blank})/ (A_{test} - A_{control})] \times 100 \quad (3.2)$$

Where A_{sample} is the absorbance of the mixture of extract, starch and enzyme solution; A_{blank} is the absorbance of the mixture of extract, starch solution but without extract; A_{test} is the absorbance of the mixture of starch and enzyme mixture; $A_{control}$ is the absorbance of the mixture of extract, starch solution mixture without legume extract fractions.

3.2.10 α-Glucosidase inhibition assay

Yeast α-glucosidase inhibitory activity was determined according to earlier reported method with slightly modification (Zhang and others 2011). In brief, 80 µL of each sample solution with appropriate concentrations were mixed with 100 µL of 4 mM 4-nitrophenyl β-D-glucuronide (pNPG) solution (dissolve in 0.1 M pH 6.8 phosphate buffer) in 1.5 mL centrifuge tube, and 20 µL of the 1U/mL enzyme solution were added to start the reaction at 37 ^{o}C for 10 min. After 10 min, 200 µl of the mixture were taken and added into wells of 96 well plate, and the release of p-nitrophenol from pNPG was measured at 405 nm. The percentage of inhibition was calculated by the following equation. The inhibition rate was determined at five different concentrations of samples to get IC_{50} (µg/mL).

$$\alpha\text{-Glucosidase inhibition\%} = [(A_{sample} - A_{blank}) / (A_{test} - A_{control})] \times 100 \quad (3.3)$$

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 sample and pNPG solution without enzyme solution; A_{test} is the absorbance of the mixture of buffer instead of sample, pNPG

solution with enzyme solution; $A_{control}$ is the absorbance of the mixture of buffer and pNPG solution without enzyme solution.

3.2.11 Lipase inhibition assay

The lipase inhibition assay was conducted according to Winkler and Stuckmann (Winkler and Stuckmann 1979) with some modifications. p-Nitrophenol palmitate (pNPP) was used as substrate which was hydrolysed 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) were mixed with 50 µL pNPP in isopropanol (0.01 M) and 5 µL of legume extract in 1.5 mL centrifuge tube. Five microliters of porcine lipase enzyme solution (50 mg/mL) were added and incubated at 37 ^{o}C for exactly 5 min. Later, 200 µL of mixture were taken and added into wells of 96-well plate, the absorbance was measured at 410 nm. The percentage of inhibition was calculated by following equation. The inhibition rate was determined at five different concentrations of samples to get IC_{50} (mg/mL).

$$\text{Lipase inhibition\%} = [(A_{sample} - A_{blank}) / (A_{test} - A_{control})] \times 100 \qquad (3.4)$$

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 sample, gum solution but without extract; A_{test} is the absorbance of the mixture of buffer instead of sample, gum solution and enzyme mixture; $A_{control}$ is the absorbance of the mixture of sample, gum mixture without legume extract or fractions.

3.2.12 Data analysis

Data analyses were carried out using a completely randomized design. Each assay was carried out in triplicate. The data were analyzed by ANOVA using 2014 SAS (version 9.3, SAS Inc., Cary, N.C, U.S.A.). Duncan's multiple range test was carried out to determine any significant differences between different samples and fractions (α=0.05).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening for the phenolic substances in eight types of common foods

Eight types of foods were initially used in this study for comparison: black bean, black soybean, black tea, green tea, blueberry, blackberry, red cabbage and broccoli. Total phenolic content, total flavonoids content, condensed tannins content, antioxidant capacity, and the IC_{50} values of crude and semi-purified extracts against α-amylase, α-glucosidase and lipase were measured.

Table 4.1 shows that TPC of all foods concentrated by the XAD-7 column, with red cabbage and broccoli having the highest purification fold for TPC and TFC, whereas red cabbage, black soybean and black bean had the highest CTC purification fold than others. Among all foods, tea had the lowest purification fold, this might be due to the saturation of the column with tea phenolics in the crude extract, which had the highest TPC content, 17-20% on a dry weight basis.

Table 4.1 Total phenolic content, total flavonoid content and condensed tannin content of crude and semi-purified extracts from eight types of foods

	Total Phenolic Content (mg GAE/g)			Total Flavonoid content (mg CE/g)			Condensed Tannin Content (mg CE/g)		
	Crude Extracts	Semi-purified Extracts	Fold[a]	Crude Extracts	Semi-purified Extracts	Fold	Crude Extracts	Semi-purified Extracts	Fold
Black tea	174.69±5.96b	319.72±10.41d	1.8	88.91±2.39a	111.61±0.94f	1.3	67.36±1.22b	94.39±9.91f	1.4
Green tea	201.43±4.72a	501.41±10.11a	2.5	81.80±1.43b	105.09±2.24g	1.3	78.23±1.62a	129.75±8.84e	1.7
Black bean	60.03±2.08e	331.41±16.16c	5.5	70.21±0.53c	174.78±0.51b	2.5	40.69±0.75e	354.75±22.9a	8.7
Black soybean	40.07±1.41f	227.78±1.03f	5.7	49.64±0.31d	139.78±1.52d	2.8	20.64±0.12f	187.38±7.95d	9.06
Red cabbage	4.99±0.24g	293.57±9.09e	58	1.73±0.13f	124.78±3.54e	72	1.28±0.12g	14.75±5.31g	11.5
Broccoli	2.04±0.15h	100.51±9.83g	49	0.32±0.08f	137.28±1.01d	428	1.93±0.25g	9.60±0.31g	5.0
Blue-berry	99.48±0.14c	425.07±10.89b	4.3	54.69±0.05e	221.77±2.48a	4.1	52.18±1.41c	296.32±12.10b	5.7
Black-berry	87.25±0.06d	323.57±11.11d	3.7	48.97±0.03d	164.78±0.51c	8.6	43.28±0.71d	278.51±28.30c	6.4

Data are expressed as mean ± standard deviation (n=3); values within each type of sample marked by the different letter within same column are significantly different ($P < 0.05$). [a] purification fold = semi-purified extract content/crude extract content.

In terms of the antioxidant activities (Table 4.2), purification by XDA-7 increased antioxidant capacity in all semi-purified extracts. However, the increases were the most significant for black soybean and black bean, indicating those compounds with higher antioxidant capacities were more preferentially retained when compared to that in other food extracts. Since the purification fold for CTC in the two legumes was higher than that for TPC and TFC (Table 4.1), respectively; it is possible that compounds in the CTC fractions contained high condensed tannins had high antioxidant capacity. This was observed in our previous lentil study (Zou and others 2011), that the CTC fraction from Sephadex LH-20 column had a very high antioxidant capacity.

Figure 4.1 shows that semi-purified extract of black bean had the lowest IC_{50} value against α-amylase (IC_{50} = 1.12 mg/mL). IC_{50} values of both black bean and black soybean were significantly ($P < 0.05$) decreased (58% and 28.9%, respectively), and were even lower than commercial inhibitor (IC_{50} = 2.21 mg/mL). However, IC_{50} values of black tea and green tea increased significantly (55.4% and 37.9%, respectively), the reason might be some phenolic compounds conjugated with glycoside were eluted by water. Meanwhile, IC_{50} values of blueberry and blackberry were decreased but with P value greater than 0.05. Even though the decreases of IC_{50} values were not significant, the IC_{50} values of semi-purified extracts were 39% and 29.4% lower than commercial inhibitor, respectively.

Table 4.2 ORAC and DPPH of crude extracts and semi-purified extracts from eight types of foods.

	ORAC Value (umol TEs/g)			DPPH Value (umol TE/g)		
	Crude Extracts	Semi-purified Extracts	Fold[a]	Crude Extracts	Semi-purified Extracts	Fold
Black tea	1605.64±41.81b	6427.93±36.63b	4.0	1503.28±39.28b	3016.20±41.94b	2.0
Green tea	3544.10±193.27a	7457.40±20.39a	2.1	1940.88±37.14a	3204.67±150.93a	1.7
Black bean	80.02±3.94f	3800.20±124.04e	47.5	49.25±1.35e	2660.56±68.56c	54.0
Black soybean	100.76±4.41e	4853.67±100.26d	48	23.77±0.62f	1572.72±38.34e	66.1
Red cabbage	8.79±0.49g	80.44±3.36h	9.2	31.10±2.68f	38.30±1.32g	1.2
Broccoli	8.00±0.43g	95.44±0.86g	11.9	7.23±0.45h	110.08±7.54f	15.1
Blueberry	308.73±2.82d	5535.21±261.21c	18.0	267.93±1.44d	2013.73±231.66d	7.5
Blackberry	380.44±2.91c	3520.11±144.21f	9.26	318.46±1.50c	1598.52±119.24e	5.0

Data are expressed as mean ± standard deviation (n=3); values within each type of sample marked by the different letter within same column are significantly different ($P < 0.05$). [a] purification fold = semi-purified extract content/crude extract content.

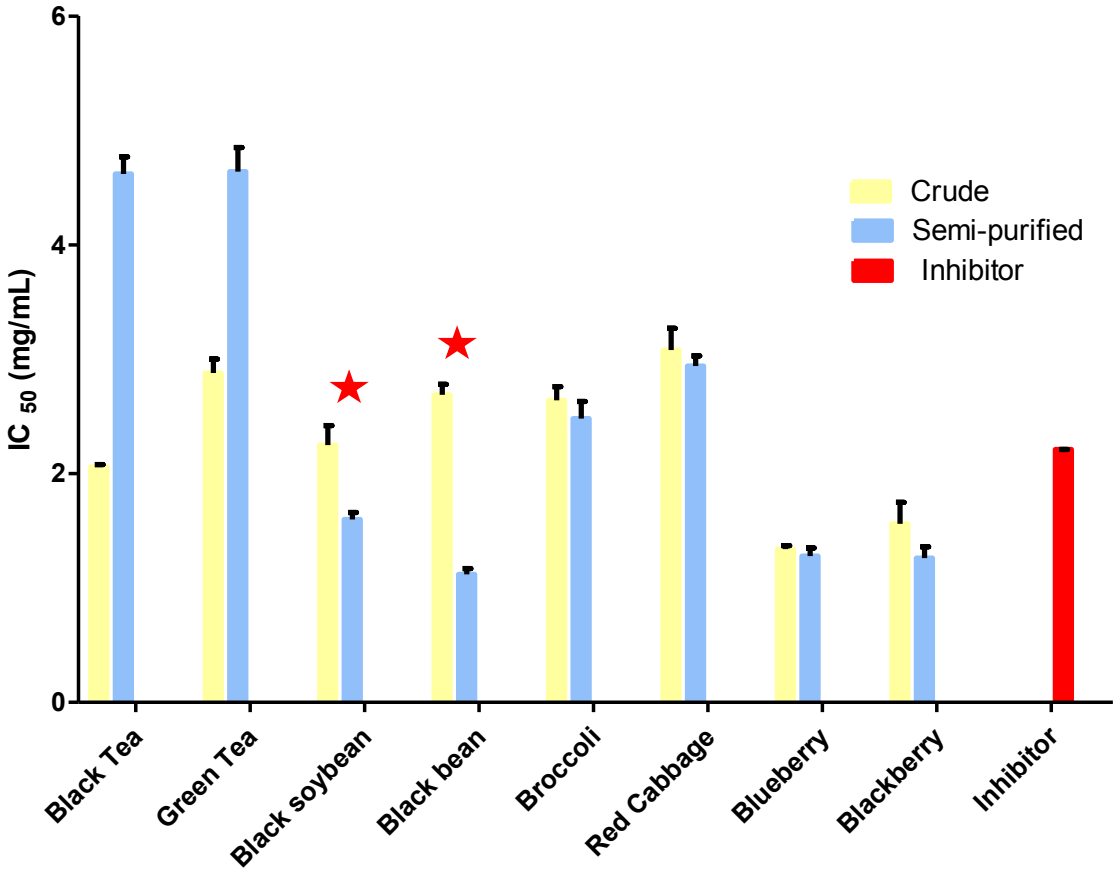


Figure 4.1 IC_{50} values of crude and semi-purified extracts from eight types of foods against α-amylase.

Star sign over bars means IC_{50} value significantly decreased compared to crude extracts ($P < 0.05$).

And for α-glucosidase inhibition (Figure 4.2), semi-purified extract of black soybean showed the lowest IC_{50} value (IC_{50} = 13.81 μg/mL). IC_{50} values of the semi-purified extracts from black bean, black soybean, broccoli and red cabbage were significantly ($p<0.05$) decreased (74.1%, 78.5%, 24% and 28.4%, respectively), and lower than commercial inhibitor (IC_{50} = 281.22 μg/mL). Meanwhile, IC_{50} values of blueberry and blackberry were decreased with P value less than 0.05, and the IC_{50} values

of semi-purified extracts were 83% and 85% lower than commercial inhibitor, respectively.

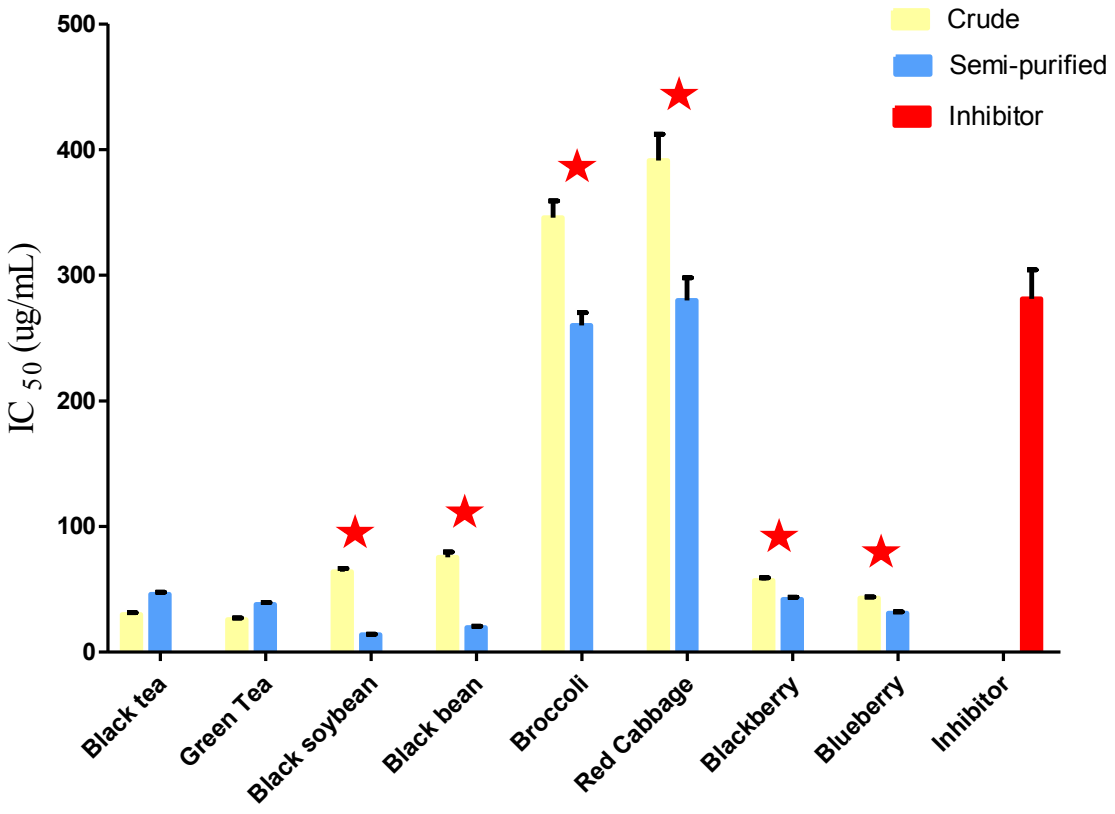


Figure 4.2 IC_{50} values of crude and semi-purified extracts from eight types of foods against α-glucosidase.

Star sign over bars means IC_{50} value significantly decreased compared to crude extracts ($P < 0.05$).

Figure 4.3 showed the IC_{50} values of crude and semi-purified extracts from eight types of foods against lipase, semi-purified extract of black soybean showed the lowest IC_{50} value (IC_{50} = 0.15 mg/mL). IC_{50} values of black bean and black soybean were significantly ($p<0.05$) decreased (41.6% and 42.3%, respectively), but still higher than commercial inhibitor (IC_{50} = 0.083 mg/mL). Meanwhile, IC_{50} values of blueberry,

blackberry, broccoli and red cabbage were decreased but with P value greater than 0.05. However, IC_{50} values of black tea and green tea increased (75% and 86.6%, respectively) significantly after eluting through XAD-7 column. The reason might be some phenolic compounds conjugated with glycoside were eluted by water, therefore some specific phenolic compounds which might possess high enzyme inhibition activity were eluted by water.

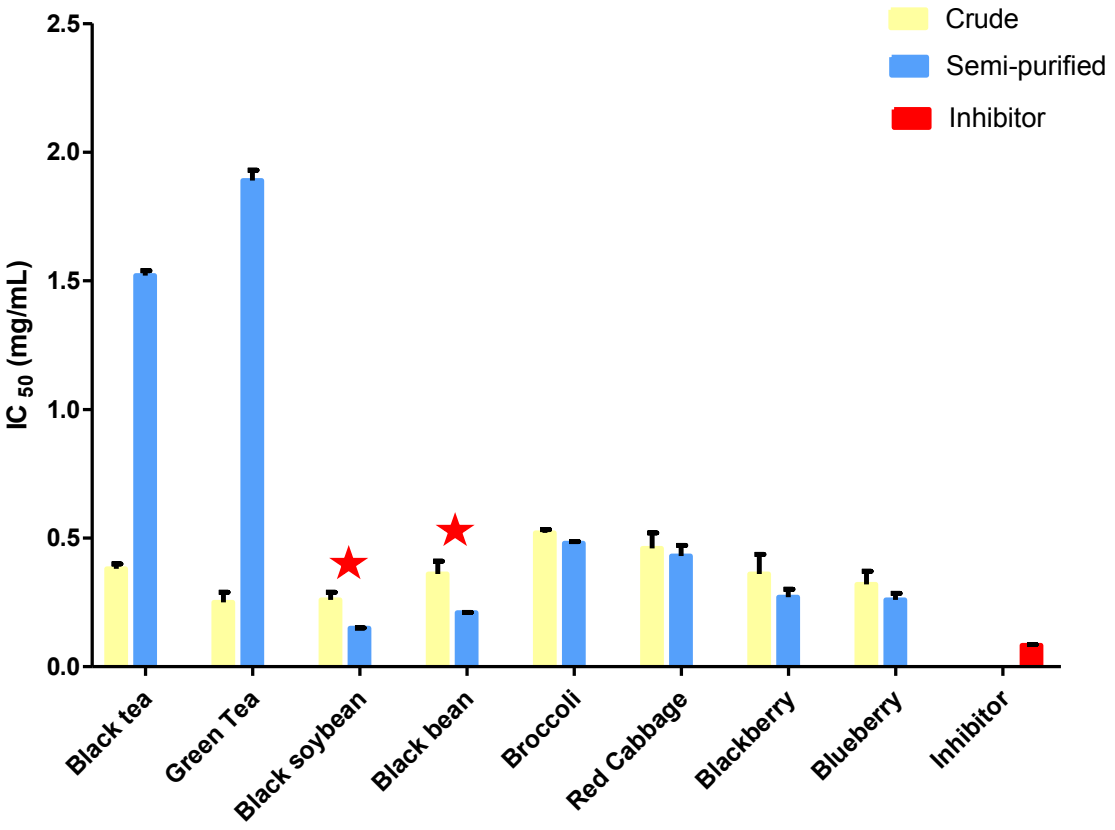


Figure 4.3 IC_{50} values of crude and semi-purified extracts from eight types of foods against lipase.

Star sign over bars means IC_{50} values significantly decreased compared to crude extracts ($P < 0.05$).

Table 4.3 Yield of the crude extracts and semi-purified extracts from eight types of foods

	Freeze dried raw material to crude extracts	Crude extracts to semi-purified extracts
Red cabbage	4.49 ± 0.13%[a]	1.5 ± 0.02%[b]
Broccoli	10.73 ± 0.23%	2.8 ± 0.03%
Black soybean	9.16 ± 0.21%	8.56 ± 0.14%
Black bean	9.45 ± 0.15%	8.86 ± 0.11%
Blueberry	2.19 ± 0.04%	4.21 ± 0.03%
Blackberry	1.7 ± 0.01%	4.6 ± 0.02%
Black tea	2.51 ± 0.03%	8.5 ± 0.31%
Green tea	2.08 ± 0.02%	19.1 ± 0.51%

[a] Based on dry sample (dry basis).
[b] Based on crude extract (freeze-dried).

Overall, the IC_{50} values of black bean, black soybean, blackberry and blueberry against α-amylase, α-glucosidase and lipase decreased after eluting through XAD-7 column; however, only black bean and black soybean significantly decreased the activity ($p<0.05$) in terms of IC_{50} values. Therefore, black bean and black soybean were selected for further fractionation. It should be noted that the IC_{50} values of red cabbage and broccoli against α-glucosidase significantly decreased after eluting from the XAD-7 column, as Figure 4.2 shows. However, the IC_{50} values still significantly greater than other foods, and therefore those two vegetables were not selected for further research. In addition, after XAD-7 column, the IC_{50} values of black tea and green tea against the three enzyme increased, the reason might be that some phenolic compounds conjugated with

glycoside, therefore some specific phenolic compounds which might possess high enzyme inhibition activity were eluted by water.

In terms of phenolic components and antioxidant activity, black bean and black soybean had significant increases in TPC, TFC, CTC, ORAC and DPPH values for the semi-purified samples, and upon the consideration of the results from IC_{50} values against α-amylase, α-glucosidase and lipase we selected black bean and black soybean for the following fractionation studies.

4.2 Extraction and fractionation

Our previous study confirmed that acidic aqueous acetone was the best system for extracting phenolics from black bean and black soybean, and this extraction solvent system gave the highest phenolic content and antioxidant activity (Xu and Chang 2007). The yields of each fraction and extract are shown in Table 4.4. Yield of crude extract was based on legume powder, the yield of semi-purified extract were based on crude extract (freeze dried), yield of fractions were based on the semi-purified extracts (freeze dried). Yield of crude extracts of black bean and black soybean were 9.45% and 9.16%, which were much higher (5.4 and 5.3%, respectively) than green lentil bean and red lentil bean (Karamac and others 2007). Genotype and different extraction solvent system might be the main reasons for the differences. Adsorption on macroporous resin and elution by sequential methanol, ethanol and acetone is a popular method for phenolic purification. The purification mainly depends on the adsorption capacity of XAD-7 for compounds with different affinity coming from different molecular weights and polarities (Silva and others 2007). The semi-purified extraction was further fractionated using Sephadex LH-20 (fractionation chromatogram are shown in Appendix A and B). The elution methods

are shown in the Figure 3.1 and 3.2. Results showed that Fraction V of black bean and black soybean were the major fractions (eluted by 600 mL 50% acetone and 600 mL 50% ethanol, respectively) among their five fractions; similar results were observed in our previous study (Zou and others 2011), the major fraction (Fraction V) was eluted by 600 mL 50% acetone.

Table 4.4 Yield of extracts and fractions of black bean and black soybean

	Black bean	Black soybean
Crude extract	9.45 ± 0.15%[a]	9.16 ± 0.21%
Semi-purified extract	8.86 ± 0.11%[b]	8.56 ± 0.14%
Fraction I	4.5 ± 0.10%[c]	3.2 ± 0.09%
Fraction II	2.7 ± 0.07%	5.2 ± 0.11%
Fraction III	2.1 ± 0.03%	1.3 ± 0.02%
Fraction IV	3.7 ± 0.04%	2.3 ± 0.03%
Fraction V	42.1 ± 1.35%	43 ± 2.01%

[a] Based on legume powder (dry weight basis).
[b] Based on crude extract (freeze-dried).
[c] Based on semi-purified extract (freeze-dried).

4.3 Total phenolic content (TPC)

Table 4.5 shows the total phenolic content (mg GAE/g) of crude extracts of black bean and black soybean, semi-purified extracts and five fractions from Sephadex LH-20 column chromatography. The total phenolic content of semi-purified extracts from black bean and black soybean were increased to 331.43 mg GAE/g and 227.86 mg GAE/g,

respectively. Generally speaking, the total phenolic content of black bean was still higher than that of black soybean. After eluting from Sephadex LH-20 column, the water elution fractions (Fraction 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 bean and 273.04 mg GAE/g for black soybean. Fraction I for both beans showed almost no phenolic compounds. Water elution fraction might contain some sugar residues or non-phenolic compounds, which have high affinity for XAD-7 resin and were not eluted with water. The total phenolic content in black bean was higher than black soybean for all of the crude extracts, semi-purified extracts and last three fractions from Sephadex LH-20 gel filtration. The reason might be that the fractions were eluted with different solvents: the last three fractions of black soybean were all eluted by 50% ethanol. However, the last fraction of black bean was eluted by 50% acetone. Thus, the polarity of the last fraction of black bean was less than that of black soybean, which means the last fraction of black bean might possess less polar compounds. In addition, total phenolic content of black bean crude extract was higher than that of black soybean (Table 4.5), which means the phenolic contents were higher in black bean due to different genotype, and this point was also illustrated by Xu and Chang (Xu and Chang 2007) that the total phenolic content of black bean was higher than that of black soybean. Overall, XAD-7 column chromatography was effective in removing sugar and organic acid (Zou and others 2011).

Table 4.5 Total phenolic content (mg GAE/g) of extracts and fractions of black bean and black soybean

	Black bean	Black soybean
Crude extract	60.03±0.28e*	40.07±0.14e
Semi-purified extract	331.43±16.16d**	227.86±10.01b
Fraction I	15.28±0.49g***	17.96±1.41f
Fraction II	75.66±1.13e	58.16±2.68d
Fraction III	363.20±2.83c	144.64±5.06c
Fraction IV	599.22±21.84a	273.04±1.13a
Fraction V	481.21±16.97b	225.44±1.69b

* Based on crude extract. ** Based on semi-purified extract. c*** Based on each fraction. Results were expressed as mean ± standard deviation (n = 3), values with different letters within a column were significantly different ($P < 0.05$).

4.4 Total flavonoid content (TFC)

Flavonoids are commonly present in plants. Epidemiological studies indicate that the consumption of flavonoid-rich foods protects against human diseases related to oxidative stress (Fraga and others 2005; Mennen and others 2004; Novotn and others 2015).

Flavonoid content (mg CE/g) in crude extracts, semi-purified extracts and fractions from Sephadex LH-20 column chromatography are shown in Table 4.6. The TFC in semi-purified extracts was much higher than that of crude extracts. After Sephadex LH-20 column chromatography, water-eluted fractions contained almost no flavonoid content especially Fraction I in comparison with last three fractions and the

similar results were observed in our previous study (Zou and others 2011). For black bean, the highest TFC was found in Fraction V, which contained 295.31mg CE/g, followed by Fraction IV, semi-purified extract and Fraction III. In terms of black soybean, the highest TFC was found in Fraction V, containing 189.00 mg CE/g, followed by semi-purified extract, Fraction III and Fraction IV, however, in our previous study, Fraction IV of lentil from Sephadex LH-20 was 367.7 mg CE/g (Zou and others 2011). Overall, TFC distribution has the similar pattern to total phenolic content in this two legumes and black bean had higher TFC than black soybean for both extracts and last three fractions.

Table 4.6 Total flavonoids content (mg CE/g) of extracts and fractions of black bean and black soybean

	Black bean	Black soybean
Crude extract	70.21±0.53e*	49.64±0.31e
Semi-purified extract	174.78±0.51c**	139.78±1.52b
Fraction I	15.31±3.09g***	19.28±1.37f
Fraction II	59.38±0.88f	61.31±2.13d
Fraction III	143.33±1.44d	139.25±1.06b
Fraction IV	281.25±3.54b	110.75±1.96c
Fraction V	295.31±2.21a	189.00±0.71a

*Based on crude extract. ** Based on semi-purified extract. *** Based on each fraction. Results were expressed as mean ± standard deviation (n = 3), values with different letters within a column were significantly different ($P < 0.05$).

4.5 Condensed tannin content (CTC)

Tannins are produced by condensation of simple phenolics and have many variations in molecular structures. Condensed tannins are the main phenolic compounds in legume seeds and occur in lentil, pea, common bean, colored soybean (Amarowicz and others 2010; Beninger and Hosfield 2003; Price and others 1980; Troszynska and Ciska 2002; Xu and Chang 2008). Condensed tannins are mainly found in the seed coat of legumes, and can protect legume from oxidative damage by some environmental factors (Troszynska and Ciska 2002).

Condensed tannin contents of extracts and fractions from black bean and black soybean are presented in Table 4.7. Condensed tannins are relatively high molecular weight compounds and can be eluted by acetone. No condensed tannin content was detected in Fraction I and II for both black bean and black soybean. For black 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) among fractions from black soybean, which means this fraction was mainly composed of condensed tannin other than phenolic acids or flavonoids. With the similar purification method we previously reported, the highest CTC was found in the last fraction of lentil which contained 744.5 mg CE/g, and the Fraction III only contained 96.5 mg CE/g (Zou and others 2011); and in our small red bean study, the last fraction of small red bean contained 591.6 mg CE/g (Zou and Chang 2014). However, the CTC values in Fraction V of black bean and Fraction IV of black soybean were higher than that of in the last fraction of lentils (906.32 mg CE/g and 797.53 mg CE/g, respectively). In terms of

determination method, the color reaction might be caused by the catechin or other monomeric flavanols which reacted with vanillin and HCl reagent, and therefore, the condensed tannin content might be overestimated.

Table 4.7 Condensed tannin content (mg CE/g) of extracts and fractions of black bean and black soybean

	Black bean	Black soybean
Crude extract	40.69±0.75e*	20.64±0.12e
Semi-purified extract	354.75±22.91d**	187.38±7.95d
Fraction I	ND	ND
Fraction II	ND	ND
Fraction III	571.21±14.14c***	670.50±70.70b
Fraction IV	691.34±19.41b	797.53±42.43a
Fraction V	906.32±63.64a	600.51±7.07c

*Based on crude extract. ** Based on semi-purified extract. *** Based on each fraction. Results were expressed as mean ± standard deviation (n = 3), values with different letters within a column were significantly different ($P < 0.05$).

4.6 Antioxidant activity of extractions and fractions

It is not appropriate to evaluate the antioxidant activity with one single method due to the complicated multi-functional nature of phytochemicals and the antioxidant activity determination methods are based on different mechanisms (Prior and others 2005). Numerous antioxidant methods have been developed to determine the antioxidant activity, among which, DPPH assay and oxygen radical absorbance capacity (ORAC) are most commonly used methods to evaluate the antioxidant activity of foods. DPPH assay

is based on the electron transfer mechanism, in which when accepting electron from an antioxidant, DPPH can be reduced to non-radical form from radical form. ORAC assay depends on hydrogen transfer mechanism to produce the free radical damage of the fluorescent probe, which leads to a decreasing change of fluorescent intensity; however, all antioxidants interfere with free radicals to inhibit the decrease of fluorescent probe, and antioxidative reaction mechanism of ORAC was more relevant to the free-radical elimination in a biological system (Prior and others 2005). DPPH, ORAC and FRAP assays were used to determine the antioxidant capacity of yellow bean and green bean sprouts, the results indicated that antioxidant capacity trends for DPPH and ORAC were different, the possible reason might be that DPPH method was based on single electron transfer and ORAC method was depend on hydrogen atom transfer, therefore, the trends for ORAC and DPPH were different (Chen and Chang 2015).

The results of DPPH assay are shown in Table 4.8. The water-eluted fraction contained almost no antioxidant activity compared with Fractions III, IV and V. After separation by XAD-7 column chromatography, antioxidant acitivity of semi-purified extracts increased significantly. The Fractions I and II from Sephadex LH-20 column chromatography exhibited significantly lower antioxidant activity compared with Fractions III, IV and V. For black bean, the highest antioxidant activity was found in Fraction V (5001.38 μmol TE/g), followed by Fraction IV (4485.54 μmol TE/g), Fraction III (2660.56 μmol TE/g) and semi-purified extraction (2660.56 μmol TE/g). In terms of black soybean, Fraction IV instead of Fraction V had the highest antioxidant activity (3751.27 μmol TE/g). Fraction V of lentil eluted by Sephadex LH-20 (Zou and others

2011) presented a higher DPPH scavenging activity (5031.6 μmol TE/g) than all fractions in this study.

Table 4.8 DPPH scavenging activity (μmol TE/g) of extracts and fractions of black bean and black soybean

	Black bean	Black soybean
Crude extract	49.25±1.35f*	23.77±0.62g
Semi-purified extract	2660.56±68.56d**	1572.77±38.34d
Fraction I	51.15±12.8f***	47.35±3.82f
Fraction II	185.09±5.62e	152.09±3.61e
Fraction III	3263.82±51.19c	2622.48±32.14c
Fraction IV	4485.54±12.83b	3751.27±21.43a
Fraction V	5001.38±25.66a	2978.55±128.56b

* Based on crude extract. ** Based on semi-purified extract. *** Based on each fraction. Results were expressed as mean ± standard deviation (n = 3), values with different letters within a column were significantly different ($P < 0.05$).

The ORAC value of extracts and fractions are shown in Table 4.9. For black bean, Fraction V possessed the highest free-radical scavenging activity and reducing power (35830.26 μmol TE/g) followed by Fraction IV (31449.40 μmol TE/g) and Fraction III (21538.00 μmol TE/g). This pattern was similar to that of the total flavonoid content and condensed tannin content in black bean but different from that of the total phenolic content. However, for black soybean, Fraction IV showed the highest antioxidant activity (31932.14 μmol TE/g), followed by Fraction V (27129.27μmol TE/g). This observation was in accordance with the correlation coefficient analysis (Table 4.14) between CTC and ORAC that was relatively higher than that between TPC, TFC and ORAC. For both

of the two legumes, Fractions IV and V had higher ORAC values than our previous study (Zou and others 2011; Zou and Chang 2014).

Table 4.9 ORAC values (μmol TE/g) of extracts and fractions of black bean and black soybean

	Black bean	Black soybean
Crude extract	80.02±3.94g*	100.76±4.41g
Semi-purified extract	3800.2±124.04d**	4853.67±100.26d
Fraction I	1805.56±159.49f***	1121.42±89.24f
Fraction II	2678.28±159.18e	1789.25±128.31e
Fraction III	21538.00±91.63c	19520.80±285.41c
Fraction IV	31449.40±294.42a	31932.14±1996.82a
Fraction V	35830.26±132.61b	27129.27±1411.42b

* Based on crude extract. ** Based on semi-purified extract. *** Based on each fraction. Results were expressed as mean ± standard deviation (n = 3), values with different letters within a column were significantly different ($P < 0.05$).

4.7 α-Amylase inhibition assay

The control of postprandial plasma glucose levels is vital in the early treatment of diabetes (Monnier and others 2003). Inhibition of enzymes like α-amylase and α-glucosidase, which are involved in the carbohydrate digestion is an important method for decreasing postprandial hyperglycemia (Heo and others 2009; Kim and others 2005). α-Amylase inhibitory activity was measured at five different concentrations, and a logarithmic regression curve was established to calculate IC_{50} values. IC_{50} value represents the concentration of the drug or inhibitor that required for 50% inhibition. The results are shown in Table 4.10. All the extracts and last three fractions possessed lower

IC_{50} than commercial inhibitor (3.23 mg/mL) under our assay conditions. Water-eluted fractions from Sephadex LH-20 column chromatography showed no significant inhibition with even doses up to 2 mg/mL in the reaction.

For black bean, Fraction V possessed the lowest IC_{50}, which meant the last fractions had the highest α-amylase inhibition ability. It might be because the last fractions had the highest total flavonoid content (Table 4.6). This observation was in accordance with the correlation coefficient analysis between TFC and α-amylase inhibition activity. The correlation coefficient between TFC and α-amylase inhibition activity was relatively higher than that between TPC, CTC and α-amylase inhibition activity.

Table 4.10 IC_{50} values (mg/mL) of extracts and fractions of black bean and black soybean against α-amylase

	Black bean	Black soybean
Crude extract	2.69±0.12b*	2.25±0.011b
Semi-purified extract	1.12±0.09d**	1.60±0.008c
Fraction I	>2	>2
Fraction II	>2	>2
Fraction III	1.76±0.06c***	1.12±0.03d
Fraction IV	0.96±0.03e	0.48±0.02e
Fraction V	0.67±0.07f	0.25±0.05f
Inhibitor (from wheat seed)	3.23±0.21a	

* Based on crude extract. ** Based on semi-purified extract. *** Based on each fraction. Results were expressed as mean ± standard deviation (n = 3), values with different letters within a column were significantly different ($P < 0.05$).

For black soybean, Fraction V had the lowest IC_{50} (0.25 mg/mL) against α-amylase. However, total phenolic content of the last three fractions of black bean were higher than that of black soybean, suggesting α-amylase inhibition might be more dependent on individual phenolic compounds than TPC or antioxidant activity. IC_{50} values of methanolic extract and acetic extract from chokeberry against α-amylase were reported as 10.31 mg/mL and 13.55 mg/mL, respectively (Worsztynowicz and others 2014). Compared to these values, the legume crude extracts, semi-purified extracts and fractions were more effective than chokeberry extract. However, the extract from grape skin showed no inhibition activity against α-amylase (Zhang and others 2011). In addition, most studies used extracts to conduct α-amylase inhibition assay without a commercial inhibitor or pure phenolic compounds as a positive control, and subsequently, it makes comparing among IC_{50} values under different assay conditions difficult.

4.8 α-Glucosidase inhibition assay

α-Glucosidase inhibitory activity was measured at five different concentrations, and a logarithmic regression curve was established to calculate IC_{50} values. The results are shown in Table 4.11. All extracts and fractions except water-eluted fractions (I and II) were effective inhibitor against α-glucosidase compared with a commercial inhibitor (voglibose). For both legumes, Fraction V possessed the lowest IC_{50} value (0.25 μg/mL for black bean and 5.40 μg/mL for black soybean). High antioxidant activity of Fraction V might have contributed to the superior bioactivity. In terms of α-glucosidase inhibition activity, fractions from black bean were more effective than the corresponding fractions from black soybean. For comparison, voglibose was used as positive control, and the IC_{50} value was determined as 282.13 ug/mL under our conditions. The water-eluted fractions

showed no significant inhibition activity with even doses up to 1 mg/mL, which means the water-eluted fractions had little bioactivity in terms of α-glucosidase inhibition.

It was reported that grape skin extract had excellent α-glucosidase inhibition activity with IC_{50} of 10.5 μg/mL (Zhang and others 2011). However, Fraction V of black bean was 42 fold more effective than that of the grape skin extract, and 2 folds more effective for Fraction IV of black bean. Even the Fraction IV of black soybean was 2.5 folds more effective than the grape skin extract. To our knowledge, Fraction V of black bean is one of the strongest natural inhibitors comparable with oolong tea extract (IC_{50} = 1.34 mg/mL) and green tea extract (IC_{50} =0.735 mg/mL) (Oki and others 1999). The IC_{50} values of extract of *Barringtonia racemosa* Roxb. seeds against yeast α-glucosidase was 26.96 μg/mL (Gowri and others 2007), which also had higher IC_{50} value than the Fractions IV and V of black bean. In a latest study, phenolic profiles of 20 types of Canadian lentil were used to determine the IC_{50} values against α-glucosidase, and the IC_{50} values were all higher than 20 mg/mL (Zhang and others 2015), which were significantly higher than all of our extracts and fractions.

Natural α-amylase and α-glucosidase inhibitors from food sources provide an appealing strategy to control post-prandial hyperglycemia. Inhibitors from food sources had lower inhibitory ability against α-amylase and stronger inhibition activity against α-glucosidase which can minimize the side effects such as abdominal distention and flatulence (Kown and others 2006). Over all, the potent α-glucosidase inhibitory activity of fractions from black bean demonstrated in our *in vitro* experiments needs to be substantiated *in vivo* in our future studies.

Table 4.11 IC_{50} values (μg/mL) of extracts and fractions from black bean and black soybean against yeast α-glucosidase

	Black bean	Black soybean
Crude extract	64.12±2.12b*	75.41±3.11b
Semi-purified extract	13.81±0.83c**	19.52±1.08c
Fraction I	>1000	>1000
Fraction II	>1000	>1000
Fraction III	8.03±0.46d***	25.01±1.33d
Fraction IV	3.28±0.13e	13.92±1.02e
Fraction V	0.25±0.07f	5.41±0.045f
Inhibitor (voglibose)	281.22±12.21a	281.22±12.21a

* Based on crude extract. ** Based on semi-purified extract. *** Based on each fraction. Results were expressed as mean ± standard deviation (n = 3), values with different letters within a column were significantly different ($P < 0.05$).

4.9 Lipase inhibition assay

Lipase inhibitory activity was measured at five different concentrations, and a logarithmic regression curve was established to calculate IC_{50} values. Results are shown in Table 4.12. All extracts and fractions except water-eluted fractions were effective inhibitors of pancreatic lipase *in vitro* compared with commercial pancreatic lipase inhibitor. For both samples, Fraction V possessed the lowest IC_{50} value (0.076 mg/mL for black bean and 0.081 mg/mL for black soybean). The water-eluted fractions showed no significant inhibition activity even with doses up to 0.5 mg/mL in the reaction. Fraction

IV of black soybean possessed high content of condensed tannins, and this might be the reason why Fraction IV had the lowest IC_{50} among black soybean fractions.

It had been reported that tannin-rich berry extract possessed high lipase inhibition activity (McDougall and others 2009). Researchers contributed this inhibition activity to tannin structures, which had lipase binding affinity (Sugiyama and others 2007). Fraction V of black bean showed the highest condensed tannin content; however it didn't exhibit the lowest IC_{50} value, suggesting condensed tannin content was not the only factor for the outstanding lipase inhibition activity. It is likely some specific tannin structures were more effective for lipase inhibition. Therefore, the amount of condensed tannin alone might not be a very appropriate indicator for lipase inhibition ability. It had been reported that methanol extract from chokeberry had lipase inhibition activity with IC_{50} value of 83.45 mg/mL (Worsztynowicz and others 2014). Epigallocatechin 3-O-gallate (EGCG), which was one of the major polyphenols in green tea, showed lipase inhibition with IC_{50} of 0.349 μM (0.159 mg/mL equivalent) (Nakai and others 2005). Recently, phenolic extracts from twenty types of lentil were used to determine the IC_{50} values against lipase, and the results indicated that the IC_{50} values of all these phenolic extracts against lipase were higher than 6 mg/mL (Zhang and others 2015) , which were significantly higher than that of this study. However, they did not use commercial inhibitors as positive control. Therefore, it is difficult to compare IC_{50} values fairly, which resulted from different assay conditions and raw materials.

Table 4.12 IC_{50} values (mg/mL) of extracts and fractions of black bean and black soybean against lipase

	Black bean	Black soybean
Crude extract	0.38±0.02a*	0.27±0.011a
Semi-purified extract	0.30±0.014b**	0.25±0.008a
Fraction I	>0.5	>0.5
Fraction II	>0.5	>0.5
Fraction III	0.26±0.013c***	0.21±0.009b
Fraction IV	0.076±0.006e	0.081±0.009c
Fraction V	0.17±0.012d	0.19±0.015b
Lipase inhibitor	0.083±0.005e	0.083±0.005c

* Based on crude extract. ** Based on semi-purified extract. *** Based on each fraction. Results were expressed as mean ± standard deviation (n = 3), values with different letters within a column were significantly different ($P < 0.05$).

4.10 Commercial pure phenolic standards against α-amylase, α-glucosidase and lipase

Since black legumes are phenolic-rich plant, 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 flavonols in black bean and black soybean (Xu and Chang 2008, 2009). Therefore, these commercial purified phenolic compounds were used to conduct the α-amylase, α-glucosidase and lipase inhibition assay. Results are shown in Figure 4.1, Figure 4.2 and Figure 4.3. The ability of the selected phenolic compounds varied significantly ($P < 0.05$).

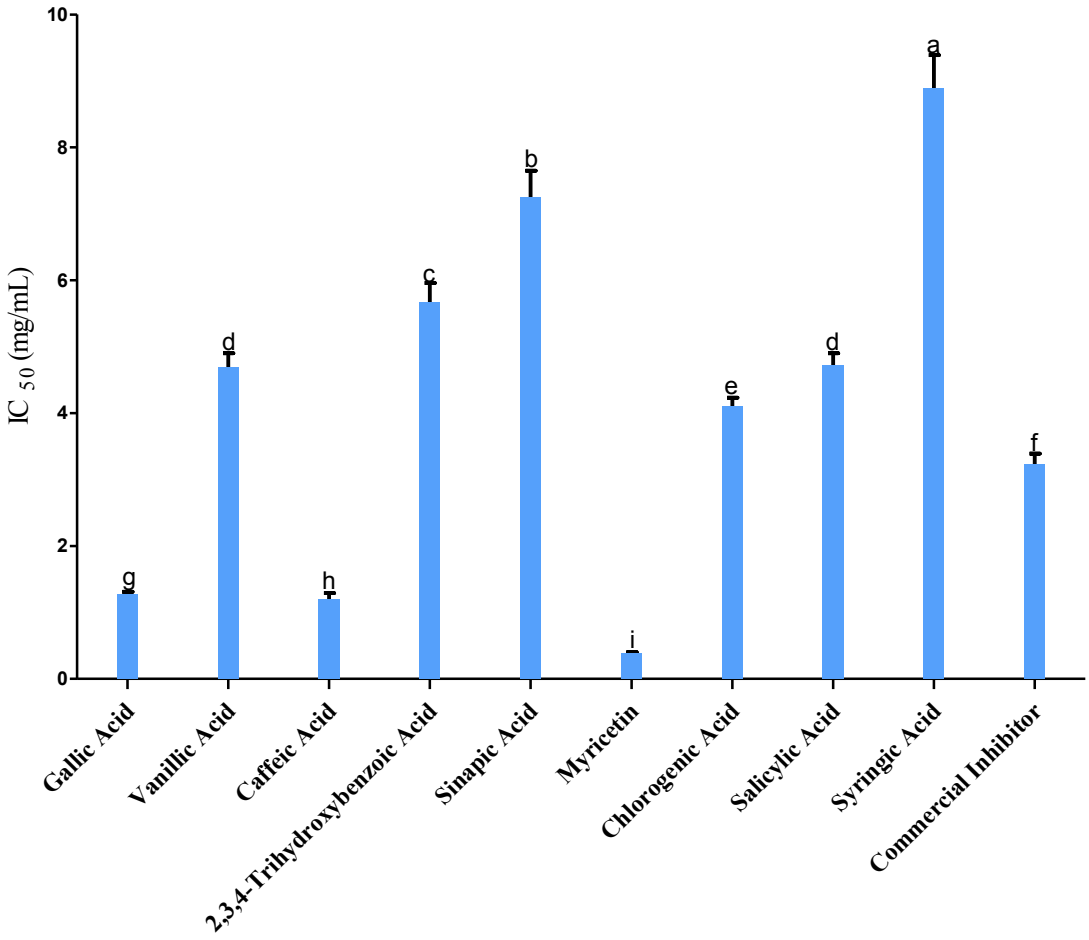


Figure 4.4 IC_{50} values of purified phenolic compounds against α-amylase. Bars marked by different letters are significantly different ($P < 0.05$).

For α-amylase inhibition, myricetin, gallic acid and caffeic acid had the highest inhibition activities (IC_{50} were 0.38 mg/mL, 1.2 mg/mL and 1.27 mg/mL, Figure 4.1), with IC_{50} values lower than the commercial α-amylase inhibitor (IC_{50} = 3.23 mg/mL). It is noteworthy that that myricetin possessed the lowest IC_{50} values against α-amylase (IC_{50} = 0.38mg/mL). Caffeic acid, coumaric acid, gallic acid and quercetin (1mg/mL) were used to determine the inhibition rate, and very low inhibition rate was observed (Apostolidis and Shetty 2008).

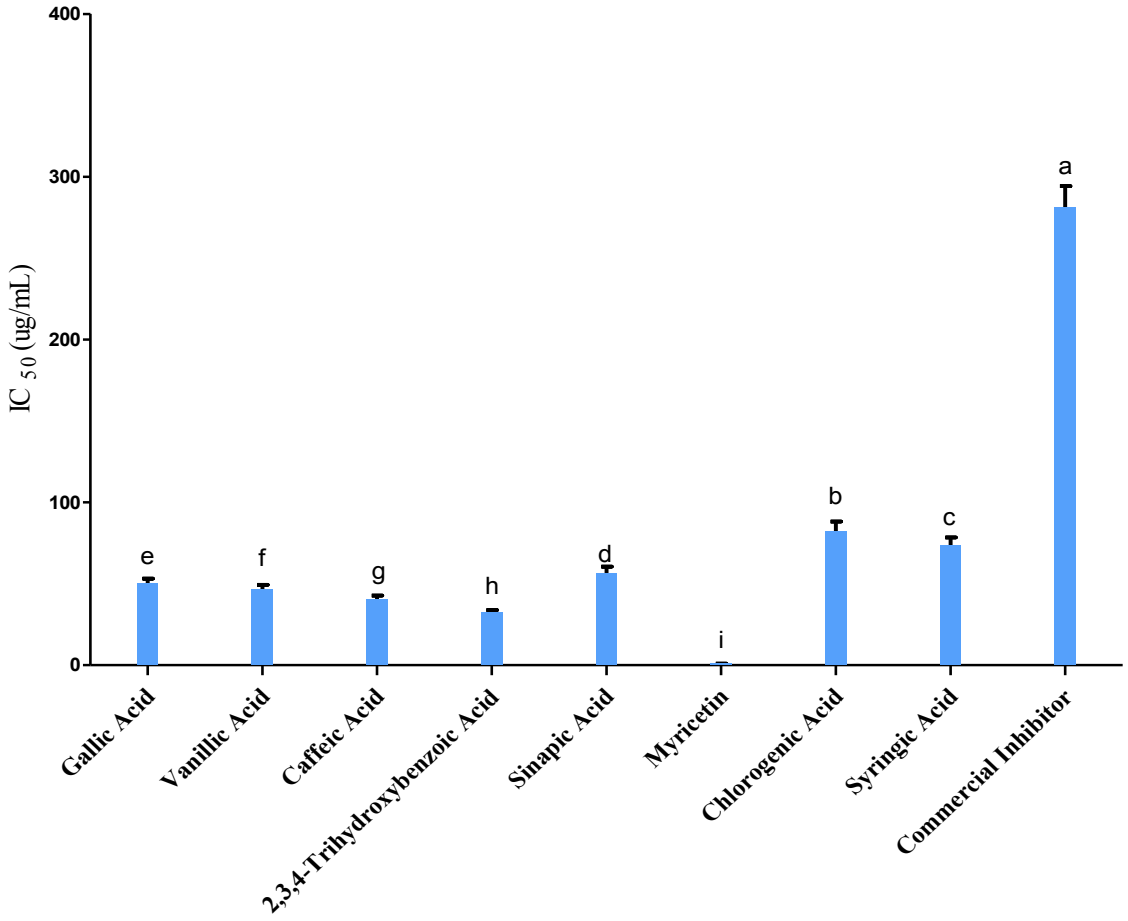


Figure 4.5 IC_{50} values of purified phenolic compounds against α-glucosidase. Bars marked by different letters are significantly different ($P < 0.05$).

For α-glucosidase inhibition activity, myricetin showed the lowest IC_{50} value (0.87 μg/mL), followed by 2,3,4-trihydroxybenzoic acid (32.16 μg/mL) and caffeic acid (40.23 μg/mL). All the commercial pure phenolic standards tested had the IC_{50} values lower than that of commercial inhibitor (281.22 μg/mL). 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 µg/mL) (Zhang and others 2015), which was higher than the IC_{50} value of myricetin (0.87 µg/mL).

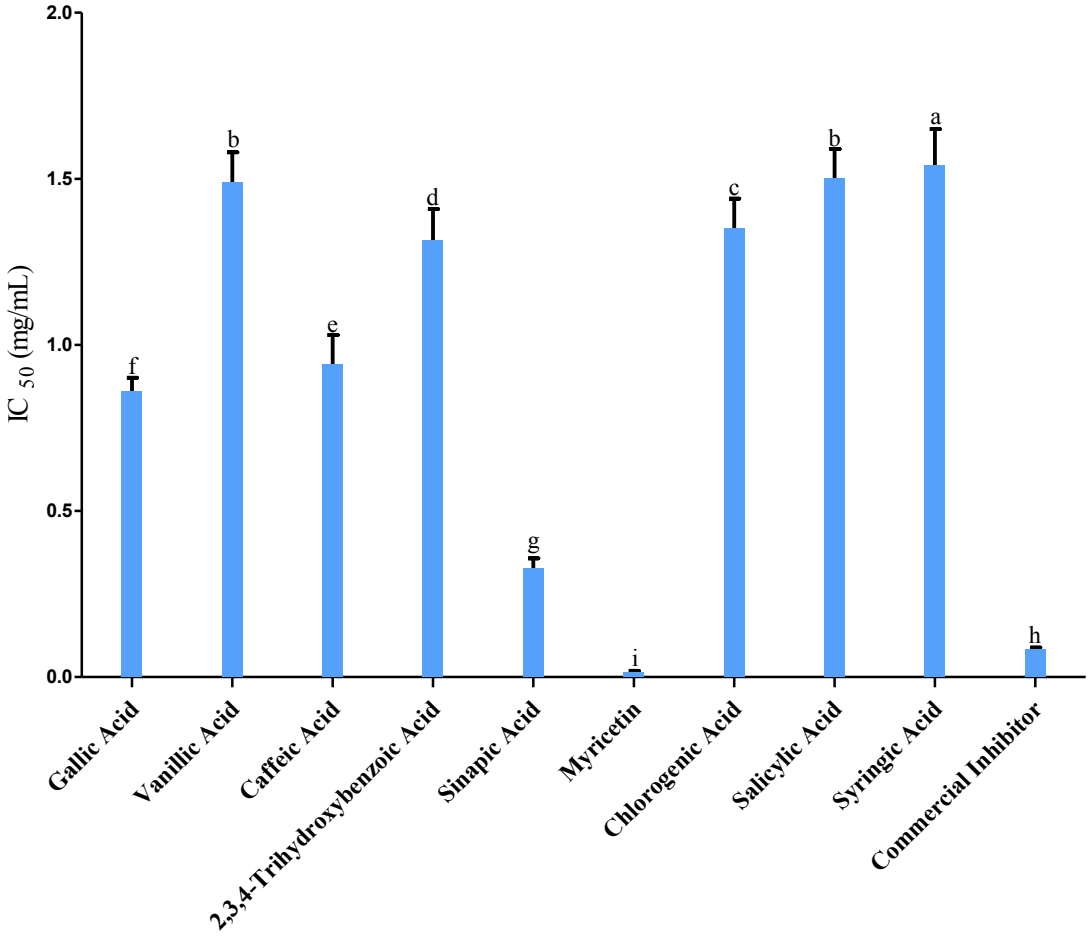


Figure 4.6 IC_{50} values of purified phenolic compounds against lipase.

Bars marked by different letters are significantly different ($P < 0.05$)

For lipase inhibition, myricetin also showed the lowest IC_{50} value (0.015 mg/mL), even lower than commercial inhibitor (0.083 mg/mL). Among all pure phenolic standards tested, myricetin was the only commercial phenolic standard that had a lower IC_{50} than commercial inhibitor. In the 20 Canadian lentil study which mentioned above (Zhang and others 2015), quercetin-arabinoside showed the lowest IC_{50} value against lipase (20.81 μg/mL) which was 27.9% higher than the IC_{50} value of myricetin (15 μg/mL) in this study.

It is noteworthy that myricetin showed the lowest IC_{50} values against α-amylase, α-glucosidase and lipase, indicating myricetin might be a good alternative phenolic compound for suppressing postprandial hyperglycemia. Nine commercial phenolic compounds showed a positive inhibition against α-amylase, α-glucosidase and lipase. However, for salicylic acid, no inhibition activity against α-glucosidase was observed. It was reported that enzymes belonging to the glycoside hydrolase family 13, such as α-amylase and α-glucosidase shared a common inhibition reaction mechanism (Inohara-Ochiai and others 1997). However, salicylic acid showed significant inhibition against α-amylase but not α-glucosidase, suggesting inhibition mechanisms of salicylic acid against α-glucosidase and α-amylase were not the same. Generally, phenolic compounds have the ability to bind to digestive enzymes to alter their activity. 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 and others 2015). However, we could not fairly compare their results with this study since the commercial inhibitor was not used, and inhibition activity was presented as percentage instead of IC_{50} value.

Recent study showed myricetin could significantly inhibit differentiation of 3T3-L1 cell from preadipocytes into adipocytes at 50 μM (Wang and others 2014), suggesting myricetin had antiobesity activity. In addition, myricetin had the function of increasing the sensitivity of insulin (Liu and others 2007), the mechanism of myricetin for increasing insulin sensitivity through improving impaired signaling intermediates of insulin receptors (Li and Ding 2012). It would be a phenolic molecule that could be increased by breeding or genetic manipulation methods for enhancing enzymatic inhibition by legumes for the management of diabetes. Overall, myricetin was a potential phenolic compound for preventing postprandial hyperglycemia and obesity.

4.11 Pearson correlation coefficient analysis

Many previous studies indicated that total phenolic content is one of the important parameters for antioxidant activity (Ismail and others 2004; Javanmardi and others 2003; Xu and others 2007). A Pearson correlation analysis was conducted to analyze the correlative relationships among antioxidant activity, phenolic substances and enzyme inhibition ability, and the results are shown in Tables 4.13 and 4.14.

For black bean, significant correlations were found among all types of phenolic contents and enzyme inhibition ability (Table 4.13). The strongest correlation was found between TPC and lipase inhibition ability ($r = -0.96$, $P < 0.01$), which suggested that total phenolic content contributed the most among those parameters to the lipase inhibition ability. Comparison of coefficients among antioxidant activity and phenolic contents revealed that significant correlations existed between all the parameters.

Table 4.13 Pearson correlation coefficient (r) among the antioxidant activity, phenolic content and enzyme inhibition ability of black bean

	TFC	CTC	DPPH	ORAC	Lipase inhibition	α-Amylase inhibition	α-Glucosidase inhibition
TPC	0.95***	0.89**	0.97***	0.89***	-0.96***	-0.88**	-0.91**
TFC		0.91**	0.96***	0.89***	-0.91**	-0.93**	-0.82*
CTC			0.98***	0.96**	-0.84*	-0.85*	-0.90**
DPPH				0.93***	-0.89**	-0.92**	-0.95**
ORAC					-0.89**	-0.71	-0.78
Lipase inhibition						0.78*	0.78*
α-Amylase inhibition							0.90**

*, significant at the 0.1 level (two-tailed); **, significant at the 0.05 level (two-tailed); ***, significant at the 0.01 level (n=24).

Condensed tannin content correlated with DPPH and ORAC the strongest, r = 0.98 and r = 0.96, respectively. This suggested that condensed tannin had contributed significantly to the antioxidant activity in black bean. In terms of α-amylase inhibition activity, among those parameters, correlation coefficients between total flavonoid content and α-amylase inhibition activity were the strongest (r = -0.93, $P < 0.05$), suggesting that flavonoid content contributed the most to α-amylase inhibition activity among those parameters. As to α-glucosidase inhibition activity, coefficient between DPPH and α-

glucosidase inhibition activity was the strongest (r = -0.95, $P < 0.05$), indicating that α-glucosidase inhibition activity was more dependent on antioxidant activity. However, coefficient between ORAC and α-glucosidase (r = -0.78, $P > 0.1$) inhibition activity was significantly lower than that between DPPH and α-glucosidase inhibition activity. In terms of mechanisms of antioxidant activity determination method, DPPH was based on electron transfer, and ORAC was based on hydrogen atom transfer (Dudonne and others 2009; Prior and others 2005). However, statistical association-ship might not be related to the chemistry of the antioxidant activities. Future research is needed to clarify the molecular mechanisms of enzyme inhibition by various phenolic substances.

For black soybean, unlike black bean, significant correlations only existed between some parameters except for between TPC and CTC, TFC and CTC, TFC and lipase inhibition activity (Table 4.14). CTC and α-glucosidase inhibition activity, ORAC and α-glucosidase inhibition ability. Similar to black bean, condensed tannin content contributed the most among those parameters to the antioxidant activity. The correlation between CTC and DPPH and ORAC were 0.96 and 0.97 ($P < 0.01$), respectively. For enzyme inhibition, correlation between α-amylase inhibition activity and ORAC and DPPH were -0.96 and -0.94 ($P < 0.05$), respectively, which meant antioxidant activity was one of the main contributors to the α-amylase inhibition ability. However, no significant correlations were observed between TPC, TFC and lipase inhibition ability. In terms of α-glucosidase inhibition activity, correlation between DPPH and α-glucosidase inhibition activity was significantly higher than that between ORAC and α-glucosidase inhibition activity. However, correlations might not be revealed the relationship among phenolic substances content, antioxidant activities and enzymes inhibition since the

sample size was small, future work is necessary to understand the mechanisms of enzyme inhibition.

Table 4.14 Pearson correlation coefficient (r) among the antioxidant activity, phenolic content and enzyme inhibition ability of black soybean

	TFC	CTC	DPPH	ORAC	Lipase inhibition	α-Amylase inhibition	α-glucosidase inhibition
TPC	0.82**	0.60	0.90*	0.80**	-0.71	-0.78	-0.91**
TFC		0.53	0.79**	0.71*	-0.22	-0.75	-0.89**
CTC			0.96***	0.97***	-0.85*	-0.88**	-0.74
DPPH				0.94**	-0.87*	-0.94**	-0.87*
ORAC					-0.89**	-0.96**	-0.75
Lipase inhibition						0.78	0.59
α-Amylase inhibition							0.87**

*, significant at 0.1 level (two-tailed); **, significant at the 0.05 level (two-tailed); *** significant at the 0.01 level (two-tailed) (n=24).

CHAPTER V

CONCLUSIONS

Crude phenolic compounds in eight types of foods were extracted by different solvent systems, and crude phenolic extracts were purified by affinity chromatography using a XAD-7 column to obtain semi-purified extracts. Black soybean and black bean were selected for further fractionation by Sephadex LH-20 column chromatography, and five fractions were obtained for both black bean and black soybean. Total phenolic content, total flavonoids content, condensed tannin content, oxygen radical absorbance capacity, radical DPPH scavenging activity, α-amylase, α-glucosidase and lipase inhibition assays were conducted for all the crude, semi-purified extracts (from eight types of foods) and fractions (from black legumes). Pure commercial phenolic standards were also used for α-amylase, α-glucosidase and lipase inhibition assay. Results indicated that Fraction V from black soybean had the lowest IC_{50} value (0.25 mg/mL) against α-amylase; Fraction V from black bean have the lowest IC_{50} value (0.25 μg/mL) against α-glucosidase; Fraction IV of black bean had the lowest IC_{50} value (76 μg/mL) against lipase. Myricetin showed the lowest IC_{50} value against α-amylase, α-glucosidase and lipase (3.23 mg/mL, 0.87 μg/mL and 15 μg/mL, respectively) among commercial pure phenolic standards even compared with the commercial inhibitors. In conclusion, several fractions obtained from Sephadex LH-20 column were more effective than commercial inhibitors of α-amylase, α-glucosidase and lipase. Among pure phenolic standards

studied, myricetin was the best for suppressing activity of α-amylase, α-glucosidase and lipase. This study contributes to the understanding of the potential of two legumes to be used for the management of diabetes. However, since legumes need to be cooked prior consumption, the retention of the phenolic compounds by cooking legumes should be studied in the future. In addition, the individual components of phenolic compounds in the fractions purified by Sephadex LH-20 also need to be studied to understand the relationships between individual components and their mixtures and effect on the inhibition of digestive enzymes linked to diabetes and obesity. Work also should be carried out using cell and animal models to test the mechanisms of inhibition in biological systems. In addition to phenolic substances, the importance of legumes carbohydrates to lower glycemic index also should be considered as part of the picture for management of type-II diabetes mellitus.

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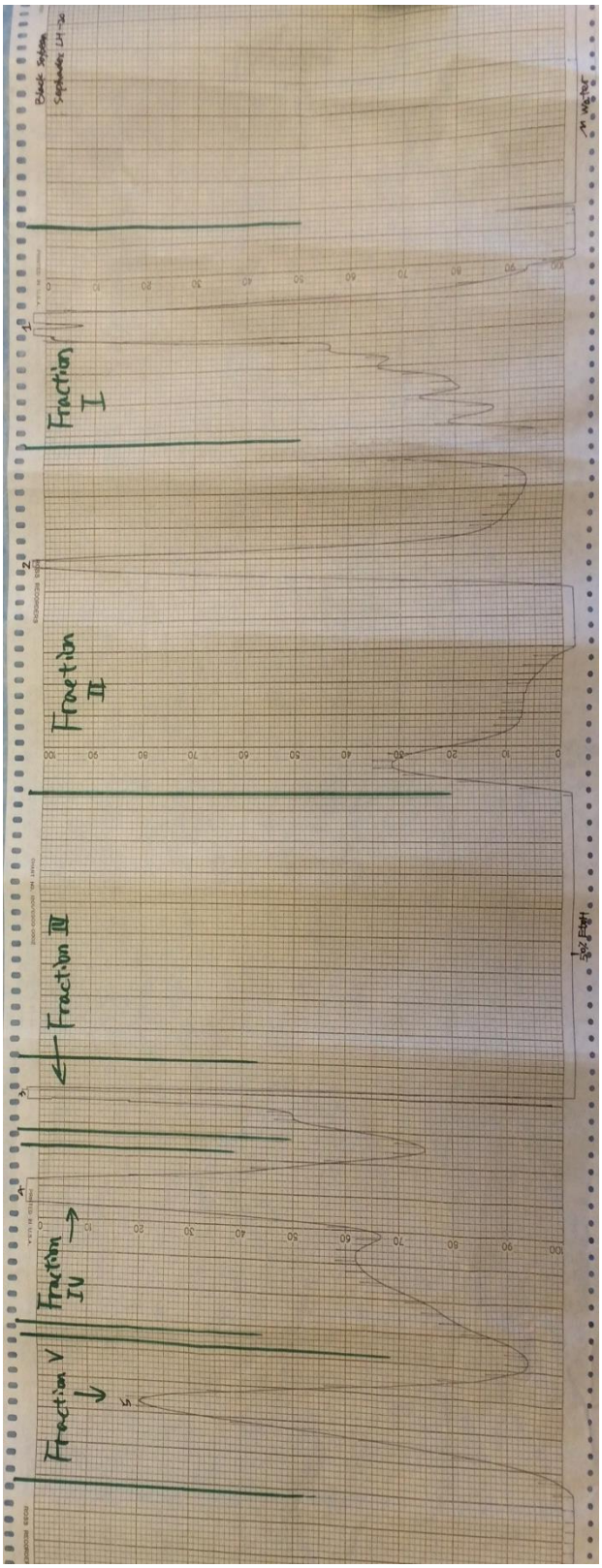
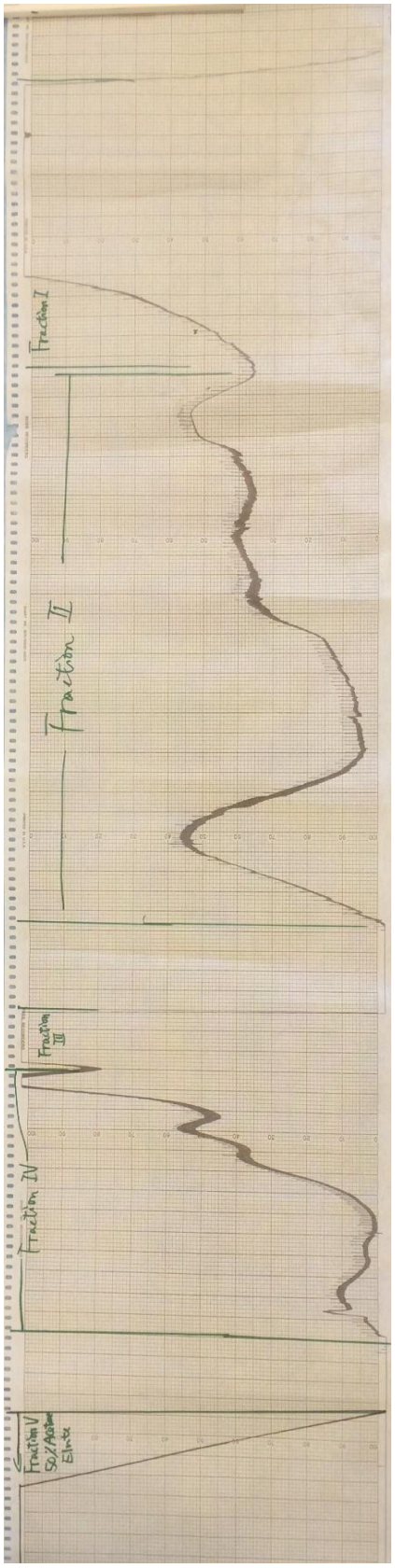
APPENDIX A

ELUTION CURVE OF FRACTIONATION OF SEMI-PURIFIED BLACK SOYBEAN EXTRACT OVER SEPHADEX LH-20



APPENDIX B

ELUTION CURVE OF FRACTIONATION OF SEMI-PURIFIED BLACK BEAN EXTRACT OVER SEPHADEX LH-20



APPENDIX C

ANOVA TABLE OF TOTAL PHENOLIC CONTENT OF EXTRACTS AND FRACTIONS FROM BLACK BEAN

The SAS System

The GLM Procedure

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	936022.8622	156003.8104	1054.37	<.0001
Error	14	2071.4328	147.9595		
Corrected Total	20	938094.2950			